EXHIBIT V

US009428548B2

(12) **United States Patent**
Brown et al.

(10) **Patent No.: US 9,428,548 B2**
(45) **Date of Patent: Aug. 30, 2016**

(54) **ENHANCED PROTEIN PURIFICATION THROUGH A MODIFIED PROTEIN A ELUTION**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 547 days.

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PCT Pub. Date: **Mar. 10, 2011**

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(51) **Int. Cl.**
C07K 1/22 (2006.01)

(52) **U.S. Cl.**
CPC *C07K 1/22* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

RE30,985 E 6/1982 Cartaya
4,515,893 A 5/1985 Kung et al.
4,560,655 A 12/1985 Baker
4,657,866 A 4/1987 Kumar
4,676,980 A 6/1987 Segal et al.
4,767,704 A 8/1988 Cleveland et al.
4,816,567 A 3/1989 Cabilly et al.
4,927,762 A 5/1990 Darfler
5,091,178 A 2/1992 Hellstrom et al.
5,091,313 A 2/1992 Chang
5,122,469 A 6/1992 Mather et al.
5,534,615 A 7/1996 Baker et al.
5,622,700 A 4/1997 Jardieu et al.
5,672,347 A 9/1997 Aggarwal et al.
5,693,762 A 12/1997 Queen et al.
5,714,338 A 2/1998 Wai Fei et al.
5,721,108 A 2/1998 Robinson et al.
5,725,856 A 3/1998 Hudziak et al.
5,736,137 A 4/1998 Anderson et al.
2006/0039901 A1 2/2006 Jiao et al.
2007/0072307 A1 3/2007 Godavarti et al.
2008/0164211 A1 7/2008 Lindner et al.
2008/0167450 A1 7/2008 Pan
2008/0193981 A1 8/2008 Fahrner et al.
2010/0331527 A1* 12/2010 Davis et al. 530/387.3

FOREIGN PATENT DOCUMENTS

EP 0 183 070 A2 6/1986
EP 0 183 070 A3 6/1986
EP 0 183 070 B1 6/1986
EP 0 244 234 A2 11/1987
EP 0 244 234 A3 11/1987
EP 0 244 234 B1 11/1987
EP 0 244 234 B2 11/1987
EP 0 308 936 A2 3/1989
EP 0 308 936 A3 3/1989
EP 0 308 936 B1 3/1989
EP 0 402 226 A1 12/1990
EP 0 420 397 B1 4/1991
RU 2145610 C1 2/2000
WO 87/00195 A1 1/1987
WO 90/03430 A1 4/1990
WO 91/00360 A1 1/1991
WO 92/20373 A1 11/1992
WO 93/04173 A1 3/1993
WO 93/08829 A1 5/1993
WO 95/19181 A1 7/1995
WO 92/23865 A1 9/1995
WO 96/27011 A1 9/1996
WO 96/30046 A1 10/1996
WO 96/40210 A1 12/1996
WO 97/26912 A2 7/1997
WO 97/26912 A3 7/1997

(Continued)

OTHER PUBLICATIONS

Hows et al. "High-performance liquid chromatography/tandem mass spectrometry assay for the rapid high sensitivity measurement of basal acetylcholine from microdialysates" J. Nueroscience methods 121, (2002) 33-39.*
Fahrner et al. "Industrial purification of pharmaceutical antibodies: development, operation, and validation of chromatography processes", Biotechnology and Genetic Engineeering Reviews, Jul. 18, 2001, pp. 301-327.*
Fahrner et al. "Industrial Purificaiton of Pharmaceutical Antibodies: development, operation, and validation of chromatography processes" Biotechnology and Genetic Engineering Reviews, 18, 2001, pp. 301-327.*
Aruffo et al., "CD44 is the Principal Cell Surface Receptor for Hyaluronate" *Cell* 61:1303-1313 (Jun. 29, 1990).
Barnes and Sato, "Methods for Growth of Cultured Cells in Serum-Free Medium" *Analytical Biochem.* 102:255-270 (1980).

(Continued)

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(57) **ABSTRACT**

The present invention provides methods for purifying a polypeptide comprising a CH2/CH3 region, comprising binding the polypeptide to Protein A and eluting with a pH gradient starting at a low pH.

73 Claims, 21 Drawing Sheets

(56) **References Cited**

FOREIGN PATENT DOCUMENTS

WO	98/06248 A2	2/1998
WO	98/06248 A3	2/1998
WO	98/23761 A1	6/1998
WO	98/45331 A2	10/1998
WO	98/45331 A3	10/1998
WO	98/51793 A1	11/1998
WO	99/01556 A2	1/1999
WO	99/01556 A3	1/1999
WO	00/75348 A1	12/2000
WO	00/75348 C1	12/2000
WO	01/00245 A2	1/2001
WO	01/00245 A3	1/2001
WO	01/40309 A2	6/2001
WO	01/40309 A3	6/2001
WO	01/40309 C1	6/2001
WO	2008/025747 A1	3/2008
WO	WO-2008/085988 A1	7/2008

OTHER PUBLICATIONS

Berg et al., "Bispecific Antibodies That Mediate Killing of Cells Infected With Human Immunodeficiency Virus of Any Strain" *Proc. Natl. Acad. Sci. USA* 88:4723-4727 (Jun. 1991).
Brennan et al., "Preparation of Biospecific Antibodies by Chemical Recombination of Monoclonal Immunoglobulin G1 Fragments" Science 229(4708):81-83 (Jul. 5, 1985).
Brodeur et al. *Monoclonal Antibody Production Techniques and Applications*. Lawrence B. Schook, New York: Marcel Dekker, Inc.,:55-63 (1987).
Brown et al., "Increasing Parvovirus Filter Throughput of Monoclonal Antibodies Using Ion Exchange Membrane Adsorptive Pre-Filtration" *Biotechnol. Bioeng.* 106(4):627-637 (2010).
Bruggermann et al., "Designer Mice: The Production of Human Antibody Repertoires in Transgenic Animals" *Year Immun* 7:33-40 (1993).
Carter et al., "High Lever *Escherichia coli* Expression and Production of a Bivalent Humanized Antibody Fragment" *Bio/Tech* 10:163-167 (Feb. 1992).
Carter et al., "Humanization of an Anti-p185^{HER2} Antibody for Human Cancer Therapy" *Proc. Natl. Acad. Sci. USA* 89:4285-4289 (May 1992).
Ceriani et al., "Biological Activity of Two Humanized Antibodies Against Two Different Breast Cancer Antigens and Comparison to Their Original Murine Forms" *Cancer Research* 55(23):5852s-5856s (1995).
Chamow et al., "A Humanized, Bispecific Immunoadhesin-Antibody That Retargets CD3$^+$Effectors to Kill HIV-1-Infected Cells" *J Immunol* 153(9):4268-4280 (Nov. 1, 1994).
Choy et al., "Percentage of Anti-CD4 Monoclonal Antibody-Coated Lymphocytes in the Rheumatoid Joint is Associated with Clinical Improvement" *Arthritis Rheum.* 39:52-56 (1996).
Clackson et al., "Making Antibody Fragments Using Phage Display Libraries" *Nature* 352:624-628 (Aug. 15, 1991).
de Wit et al., "Real-Time Quantitative PCR for Retrovirus-Like Particle Quantification in CHO Cell Culture" *Biologicals* 28:137-148 (2000).
Dhainaut et al., "CDP571, a Humanized Antibody to Human Tumor Necrosis Factor—α: Safety, Pharmacokinetics, Immune Response, and Influence of the Antibody of Cytokine Concentrations in Patients with Septic Shock" *Crit. Care Med.* 23(9):1461-1469 (1995).
Duchosal et al., "Immunization of hu-PBL-SCID Mice and the Rescue of Human Monoclonal Fab Fragments Through Combinatorial Libraries" *Nature* 355(6357):258-262 (Jan. 16, 1992).
Ellis et al. "Engineered Anti-CD38 Monoclonal Antibodies for Immunotherapy of Multiple Myeloma," *J. Immunol.* 155(2):925-937, (Jul. 15, 1995).
Goding, *Monoclonal Antibodies: Principles and Practice* "Production and Application of Monoclonal Antibodies in Cell Biology, Biochemistry and Immunology" Academic Press, 56-103 (1986).
Graham et al., "Characteristics of a Human Cell Line Transformed by DNA From Human Adenovirus Type 5" *J. Gen. Virol.* 36(1):59-72 (Jul. 1977).
Graziano et al., "Construction and Characterization of a Humanized Anti-γ-Ig Receptor Type I (FcγRI) Monoclonal Antibody" *J. Immunol.* 155:4996-5002 (1995).
Gruber et al., "Efficient Tumor Cell Lysis Mediated by a Bispecific Single Chain Antibody :Expressed in *Escherichia coli*" *J. Immunol.* 152:5368-5374 (1994).
Ham and McKeehan, "Media and Growth Requirements" *Method Enzymol.* 58:44-93 (1979).
Hollinger et al., ""Diabodies": Small Bivalent and Bispecific Antibody Fragments" *Proc. Natl. Acad. Sci. USA* 90:6444-6448 (Jul. 1993).
Hoogenboom et al., "Construction and Expression of Antibody-Tumor Necrosis Factor Fusion :Proteins" *Molecular Immunology* 28(9):1027-1037 (1991).
Hoogenboom et al., "By-Passing Immunisation. Human Antibodies From Synthetic Repertoires of Germline V_H Gene Segments Rearranged in Vitro" *J. Mol. Biol.* 227:381-388 (1992).
Hourmant et al., "Administration of an Anti-CD11a Monoclonal Antibody in Recipients of Kidney Transplantation" *Transplantation* 58(3):377-380 (Aug. 1994).
Jakobovits et al., "Analysis of Homozygous Mutant Chimeric Mice: Deletion of the Immunoglobulin Heavy-Chain Joining Region Blocks B-cell Development and Antibody Production" *Proc. Natl. Acad. Sci. USA* 90(6):2551-2555 (Mar. 15, 1993).
Jakobovits et al., "Germ-line Transmission and Expression of a Human-Derived Yeast :Artificial Chromosome" *Nature* 362(6417):255-258 (Mar. 18, 1993).
Jones et al., "Replacing the Complementarity-Determining Regions in a Human Antibody with Those From a Mouse" *Nature* 321(6069):522-525 (May 29, 1986).
Jurcic et al., "Radiolabeled Anti-CD33 Monoclonal Antibody M195 for Myeloid Leukemias" *Cancer Research* 55:5908s-5910s (Dec. 1, 1995).
Juweid et al. "Treatment of Non-Hodgkin's Lymphoma with Radiolabeled Murine, Chimeric, or Humanized LL2, an Anti-CD22 Monoclonal Antibody," *Cancer Res.* 55(23 Supp.):5899s-5907s, (Dec. 1, 1995).
Kim et al. et al., "The Vascular Endothelial Growth Factor Proteins: Identification of Biologically Relevant Regions by Neutralizing Monoclonal Antibodies" *Growth Factors*7:53-64 (1992).
Kohler and Milstein, "Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity" *Nature* 256:495-497 (Aug. 7, 1975).
Kostelny et al., "Formation of a Bispecific Antibody by the use of Leucine Zippers" *J. Immunol.* :148(5):1547-1553 (Mar. 1, 1992).
Kozbor et al., "A Human Hybrid Myeloma for Production of Human Monoclonal Antibodies" *Immunol* 133(6):3001-3005 (Dec. 1984).
Le Doussal et al., "In Vitro and In Vivo Targeting of Radiolabeled Monovalent and Divalent Haptens with Dual Specificity Monoclonal Antibody Conjugates: Enhanced Divalent Hapten Affinity for Cell-Bound Antibody Conjugate" *J. Nucl. Med.* 30:1358-1366 (1989).
Lindmark et al., "Binding of Immunoglobulins to Protein A and Immunoglobulin Levels in Mammalian Sera" *J. Immonol. Methods* 62:1-13 (1983)
Litton et al., "Antibody-Targeted Superantigen Therapy Induces Tumor-Infiltrating Lymphocytes, Excessive Cytokine Production, and Apoptosis in Human Colon Carcinoma" *Eurpoean Journal of Immunology* 26:1-9 (1996).
Lorenz, H. et al., "In Vivo Blockade of TNF-αby Intravenous Infusion of a Chimeric Monoclonal TNF-α Antibody in Patients with Rheumatoid Arthritis" *J. Immunol.* 156:1646-1653 (1996).
Marks et al., "By-Passing Immunization. Human Antibodies From V-Gene Libraries Displayed on Phage" *J. Mol. Biol.* 222:581-597 (1991).
Marks et al., "By-Passing Immunization: Building High Affinity Human Antibodies by (Chain Shuffling" *Bio/Technology* 10:779-783 (Jul. 1992).

(56) **References Cited**

OTHER PUBLICATIONS

Mather, "Establishment and Characterization of Two Distinct Mouse Testicular Epithelial Cell Lines" *Biol. Reprod*. 23:243-252 (1980).
McCafferty et al., "Phage Antibodies: Filamentous Phage Displaying Antibody Variable Domain" *Nature* 348:552-554 (Dec. 1990).
Millstein et al., "Hybrid Hybridomas and Their use in Immunohistochemistry" *Nature* 305:537-539 (1983).
Morimoto and Inouye, "Single-Step Purification of $F(ab')_2$ Fragments of Mouse Monoclonal Antibodies (Immunoglobulins G1) by Hydrophobic Interaction High Performance Liquid Chromatography Using TSKgel phenyl-5PW" *J. Biochem. Biophys. Meth*. 24:107-117 (1992).
Morrison et al., "Chimeric Human Antibody Molecules: Mouse Antigen-Binding Domains with Human Constant Region Domains" *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (Nov. 1984).
Presta, "Antibody Engineering" *Curr. Opin. Struc. Biol*. 2:593-596 (1992).
Presta et al., "Humanization of an Antibody Directed Against IgE" *J. Immunol*. 151(5):2623-2632 (Sep. 1, 1993).
Recny et al., "*N*-Glycosylation is Required for Human CD2 Immunoadhesion Functions" *J. Biol. Chem*. 267(31):428-434 (Nov. 5, 1992).
Riechmann et al., "Reshaping Human Antibodies for Therapy" *Nature* 332:323-327 (Mar. 24, 1988).
Richman et al., "Radioimmunotherapy for Breast Cancer Using Escalating Fractionated Doses of ^{131} I-Labeled Chimeric L6 Antibody With Peripheral Blood Progenitor Cell Transfusions" *Cancer Research* 55(23 Suppl):516s-5920s (1995).
Shalaby et al., "Development of Humanized Bispecific Antibodies Reactive With Cytotoxic Lymphocytes and Tumor Cells Overexpressing the *HER2* Protooncogene" *J. Exp. Med*. 175:217-225 (Jan. 1, 1992).
Sharkey et al., "Evaluation of a Complementarity-Determining Region-Grafted (Humanized) Anti-Carcinoembryonic Antigen Monoclonal Antibody in Preclinical Studies" *Cancer Research* 55(23 Suppl):5935s-5945s (1995).
Sims et al., "A Humanized CD18 Antibody Can Block Function Without Cell Destruction" *J. Immunol*151(4):2296-2308 (Aug 15, 1993).
Stamenkovic et al., "The B Lymphocyte Adhesion Molecule CD22 Interacts with Leukocyte Common Antigen CD45RO on T Cells and α2-6 Sialyltransferase, CD75, on B Cells" *Cell* 66:1133-1144 (Sep. 20, 1991).
St. John et al., "Immunologic Therapy for ARDS, Septic Shock, and Multiple-Organ Failure" *Chest* 103:932-943 (1993).
Stoppa et al., "Anti-LFA1 Monoclonal Antibody (25.3) for Treatment of Steroid-Resistant Grade III-IV Acute Graft-Versus-Host Disease" *Transplant International* 4:3-7 (1991).
Traunecker et al., "Bispecific Single Chain Molecules (Janusins) Target Cytotoxic Lymphocytes on HIV Infected Cells" *Embo J*. 10(12):3655-3659 (1991).
Tutt et al., "Trispecific $F(ab')_3$ Derivatives that Use Cooperative Signaling Via the TCR/CD3 Complex and CD2 to Activate and Redirect Resting Cytotoxic T Cells" *J. Immunol*. 147(1):60-69 (Jul. 1991).
Urlauba et al., "Isolation of Chinese Hamster Cell Mutants Deficient in Dihydrofolate Reductase Activity" *Proc. Natl. Acad. Sci. USA* 77(7):4216 (Jul. 1980).
Vaughan et al., "Human Antibodies With Sub-Nanomolar Affinities Isolated From a Large Non-immunized Phage Display Library" *Nat. Biotechnol*. 14:309-314 (Mar. 1996).
Velayudhan et al. "Modeling of Purification Operations in Biotechnology: Enabling Process Development, Optimization, and Scale-Up" *Biotechnol. Prog*. 23:68-73 (2007, e-pub. Jan. 4, 2007).
Verhoeyen et al., "Reshaping human antibodies: Grafting an Antilysozyme Activity" *Science* 239:1534-1536 (Mar. 1988).
Waterhouse et al., "Combinatorial Infection and in vivo Recombination: a Strategy for Making Large Phage Antibody Repertoires" *Nucl. Acids Res*. 21(9):2265-2266 (1993).
Zapata et al., "Engineering linear $F(ab')_2$ Fragments for Efficient Production in *Escherichia coli* and Enhanced Antiproliferative Activity" *Protein Eng*. 8(10):1057-1062 (1995).
Duhamel et al., "pH gradient elution of human IgG1, IgG2 and IgG4 from protein A-sepharose," *Journal of Immunological Methods* 31:211-217, (1979).
Martin et al., "Separation of guinea pig IgG subclasses by affinity chromatography on protein A-sepharose," *Journal of Immunological Methods* 52:205-212, (1982).
Pan et al., "Quantitation of soluble aggregates in recombinant monoclonal antibody cell culture by pH-gradient protein A chromatography," *Analytical Biochemistry* 388:273-278, (2009, e-pub. Mar. 4, 2009).
Supplementary European Search Report mailed on Mar. 28, 2014, for European Patent Application No. 10814394.2, filed on Sep. 1, 2010, 19 pages.

* cited by examiner

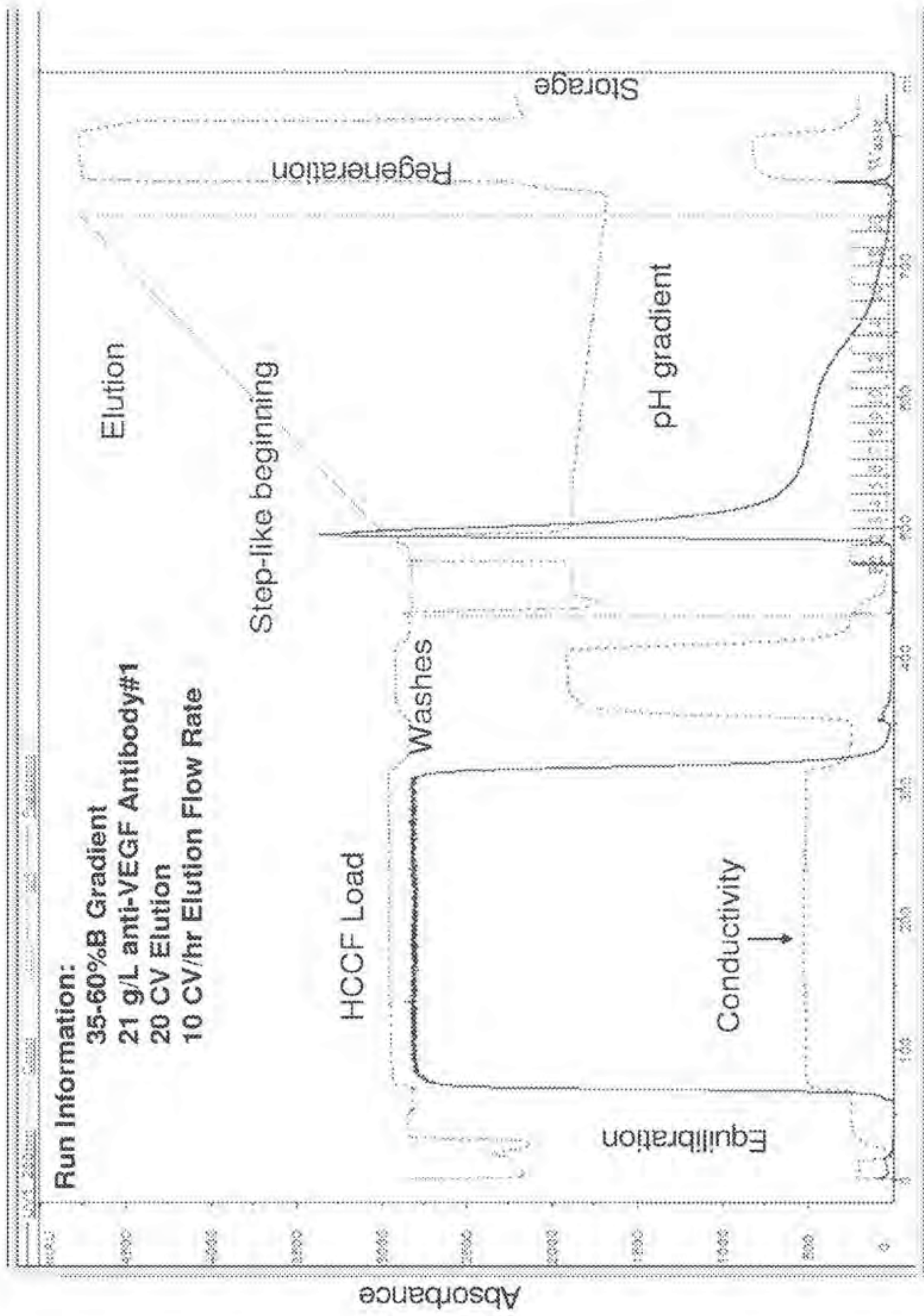

Figure 1

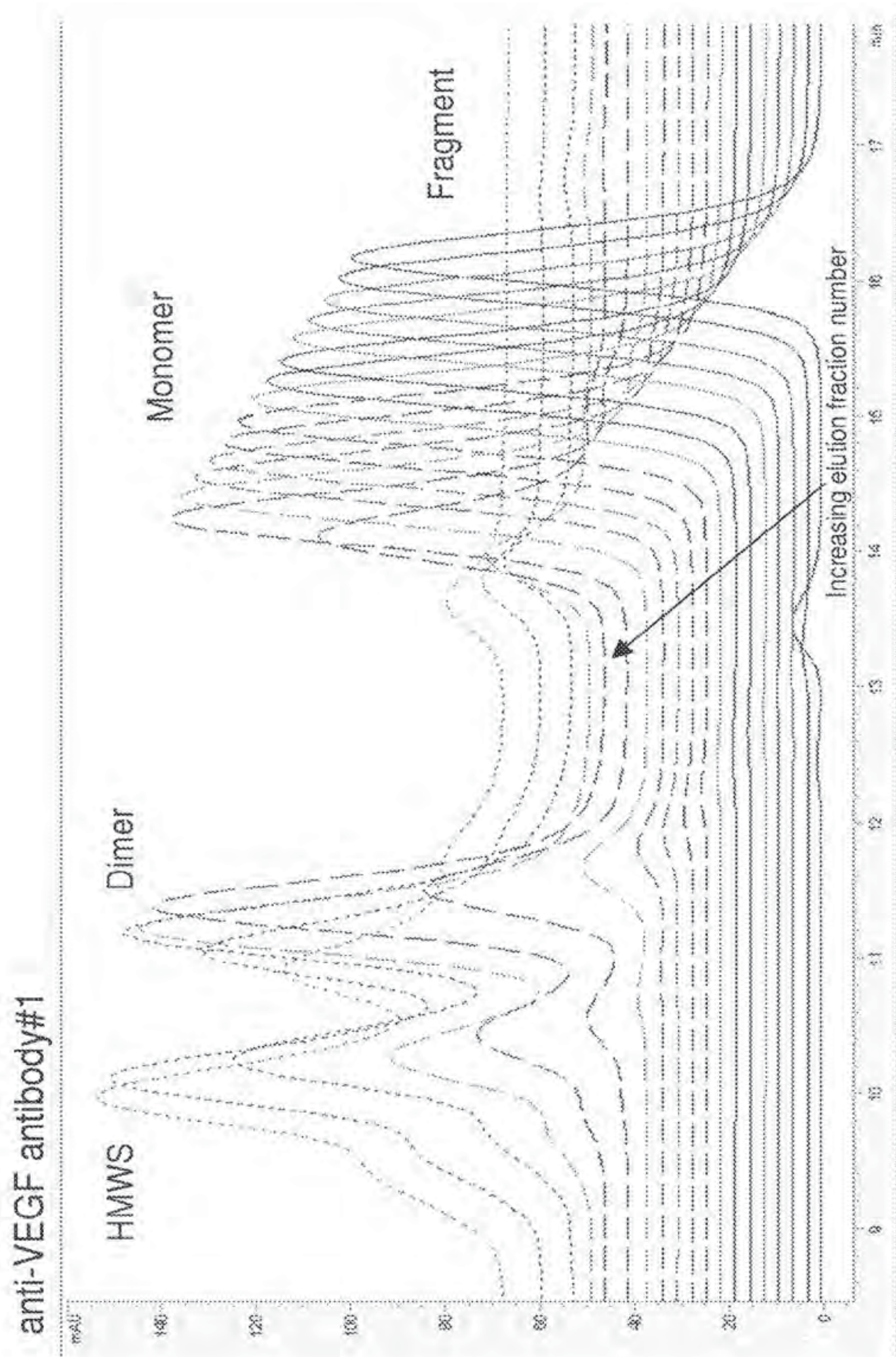

Figure 2
anti-VEGF antibody#1
HMWS
Dimer
Monomer
Fragment
Increasing elution fraction number

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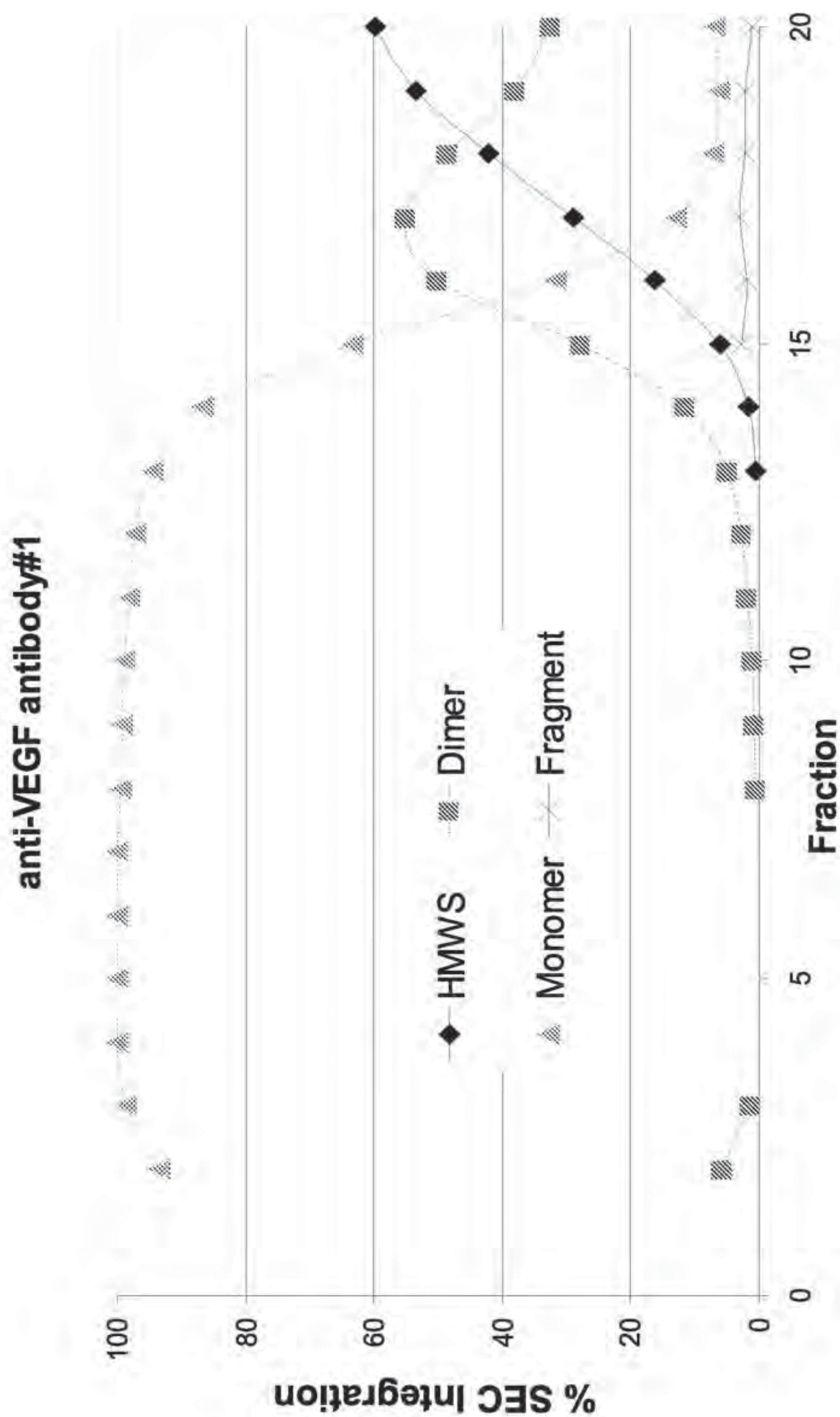
Figure 3



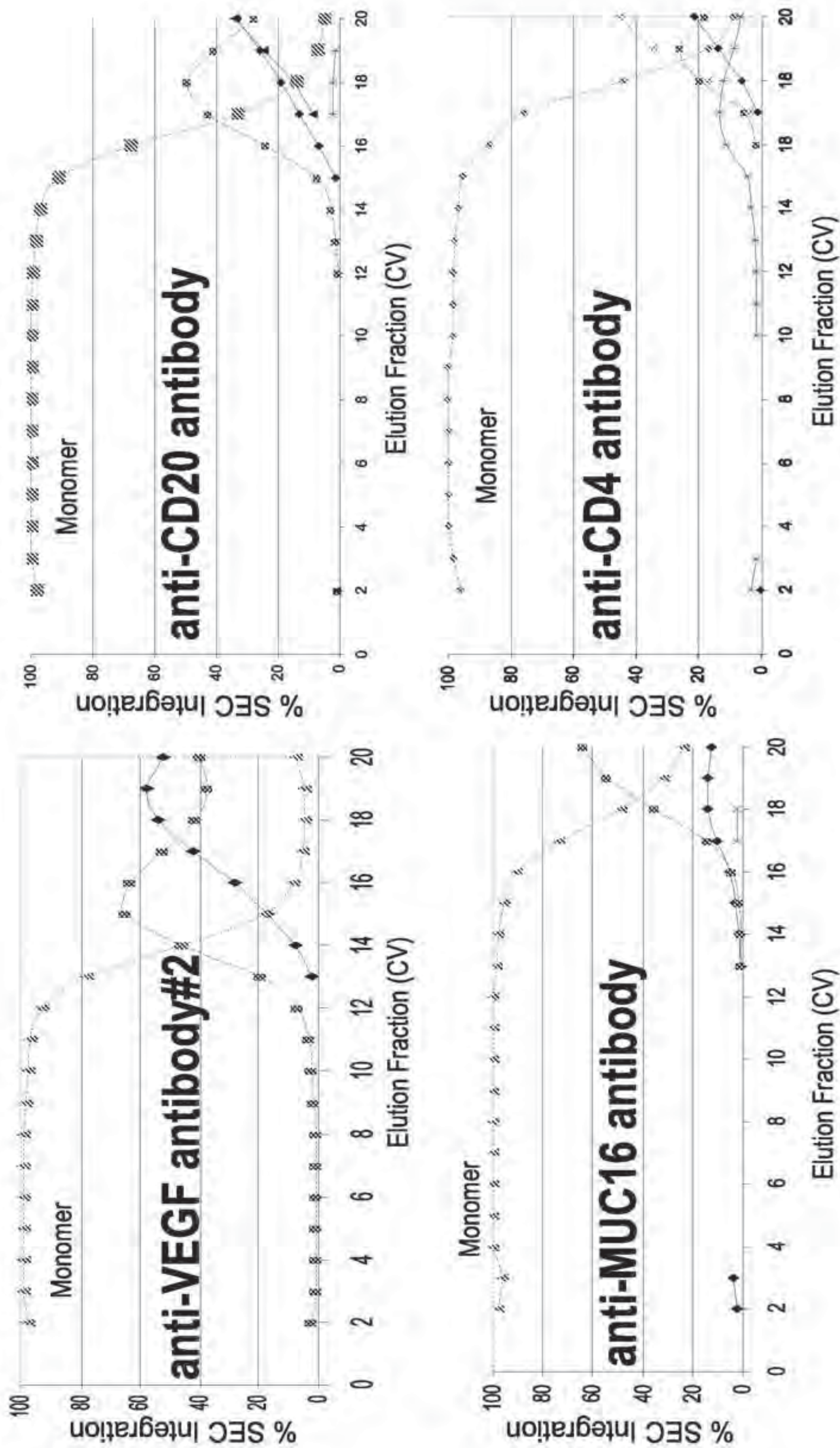
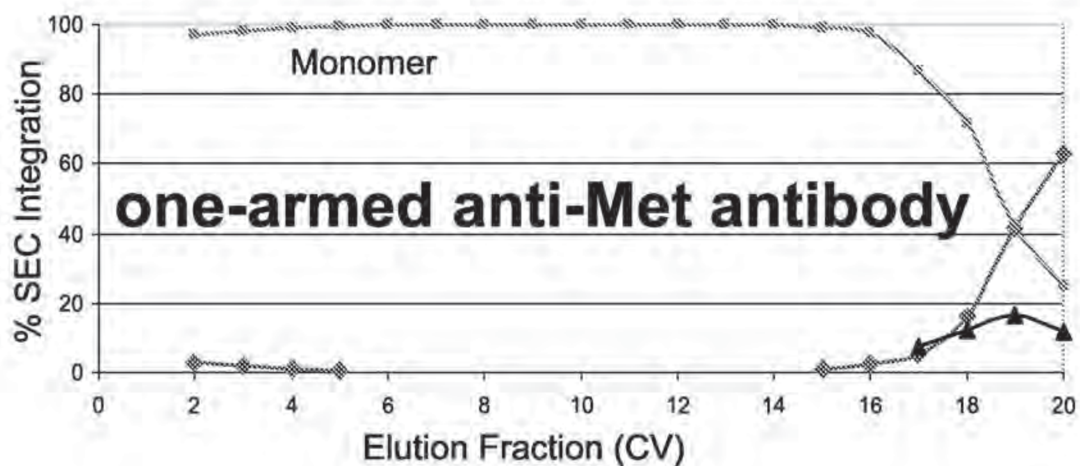
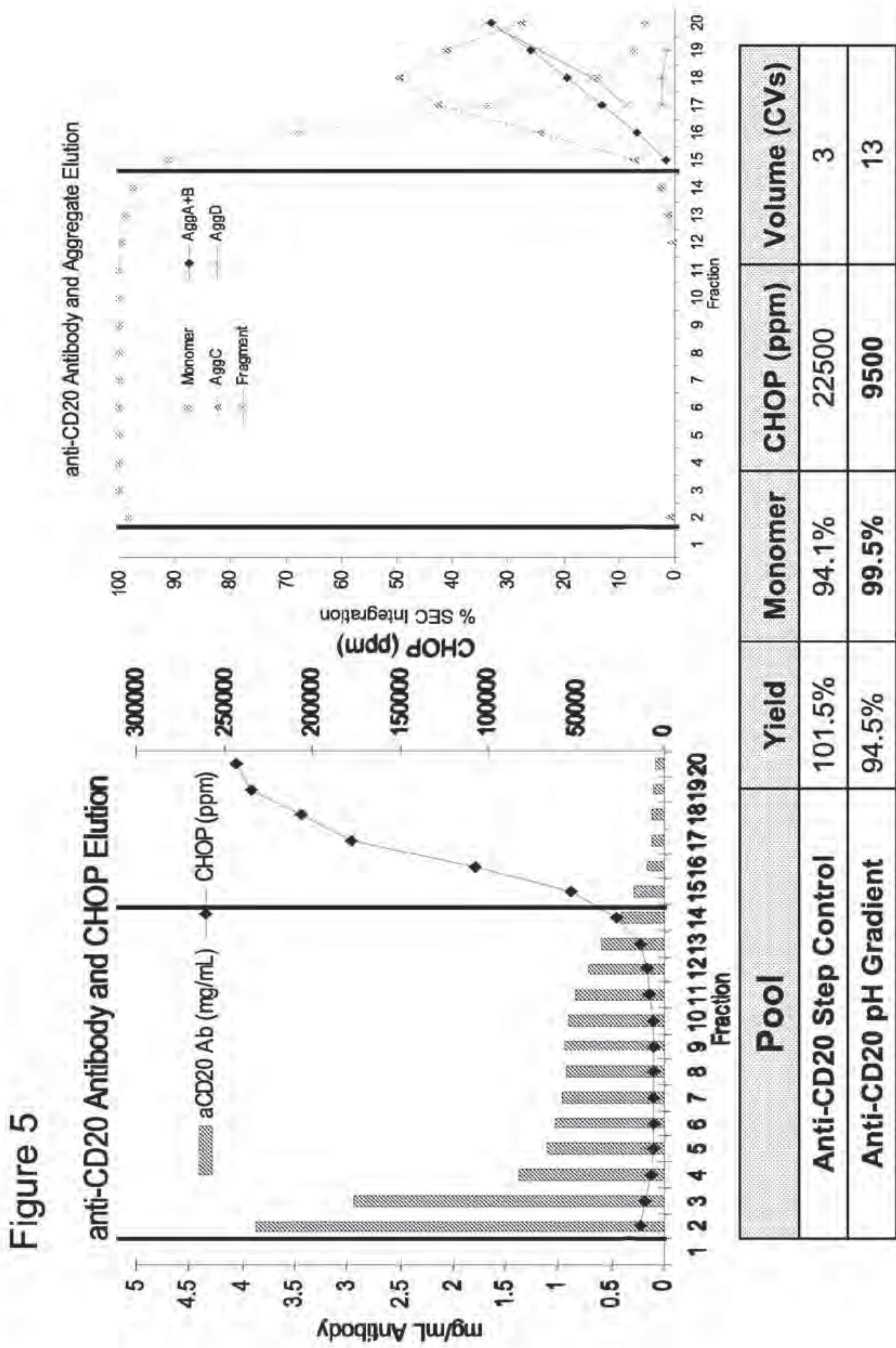


Figure 4A
anti-VEGF antibody#2
Monomer
% SEC Integration
Elution Fraction (CV)
anti-CD20 antibody
Monomer
% SEC Integration
Elution Fraction (CV)
anti-MUC16 antibody
Monomer
% SEC Integration
Elution Fraction (CV)
anti-CD4 antibody
Monomer
% SEC Integration
Elution Fraction (CV)

Figure 4B





Pool	Yield	Monomer	CHOP (ppm)	Volume (CVs)
Anti-CD20 Step Control	101.5%	94.1%	22500	3
Anti-CD20 pH Gradient	94.5%	99.5%	9500	13

* pH gradient values from calculated mock pool

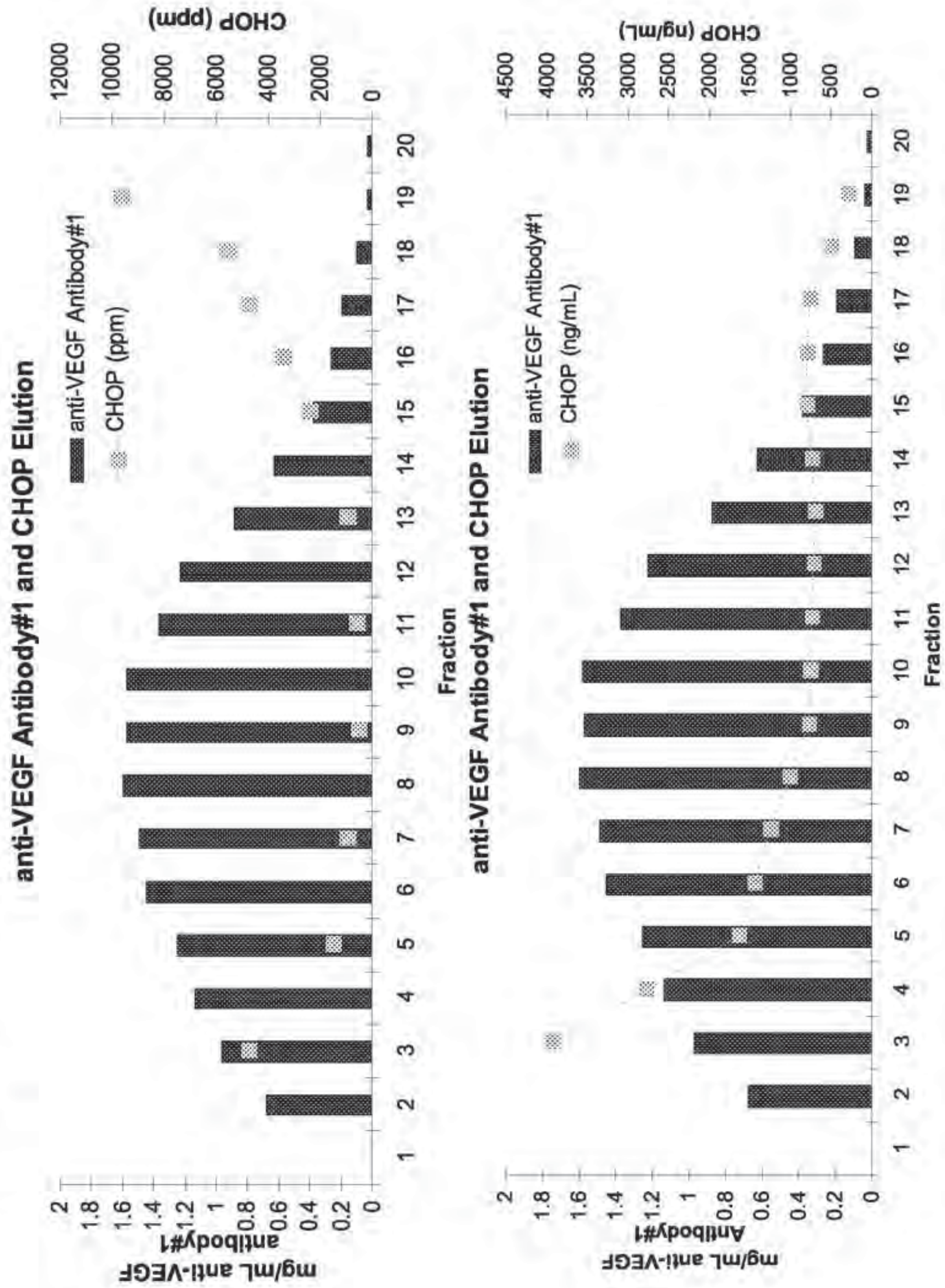
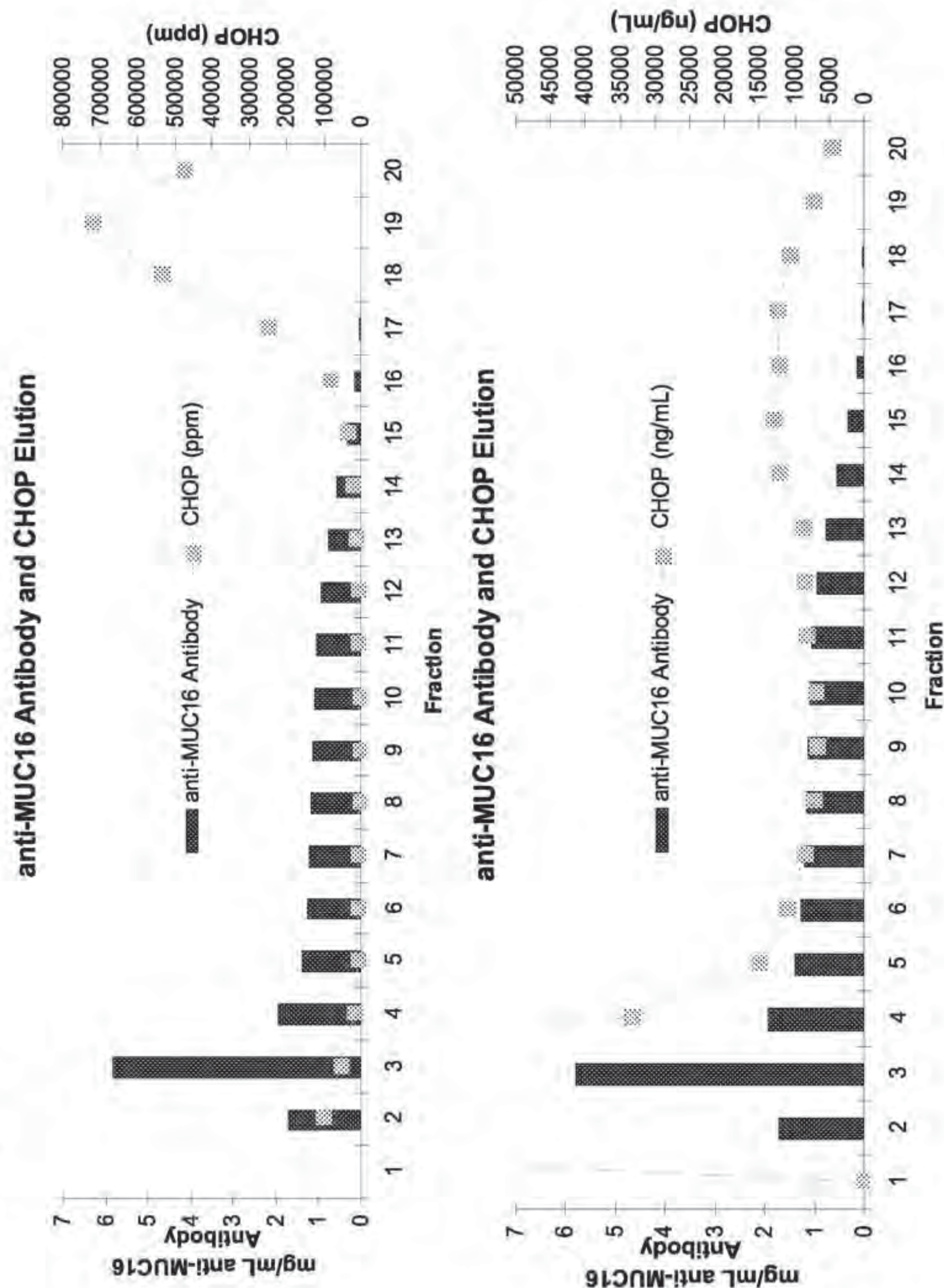
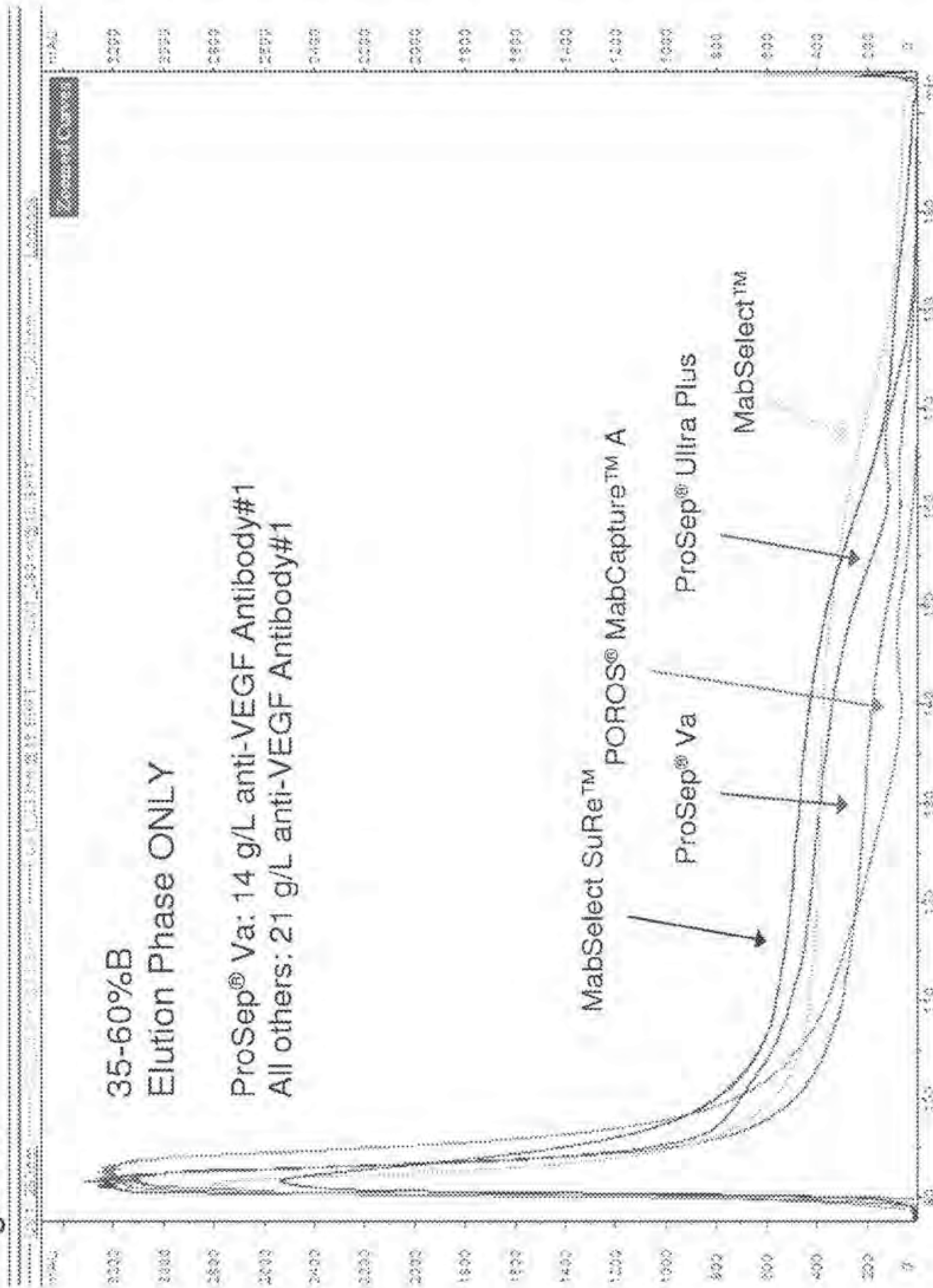


Figure 6
anti-VEGF Antibody#1 and CHOP Elution
anti-VEGF Antibody#1
CHOP (ppm)
mg/mL anti-VEGF antibody#1
CHOP (ppm)
Fraction
anti-VEGF Antibody#1 and CHOP Elution
anti-VEGF Antibody#1
CHOP (ng/mL)
mg/mL anti-VEGF Antibody#1
CHOP (ng/mL)
Fraction

Figure 7





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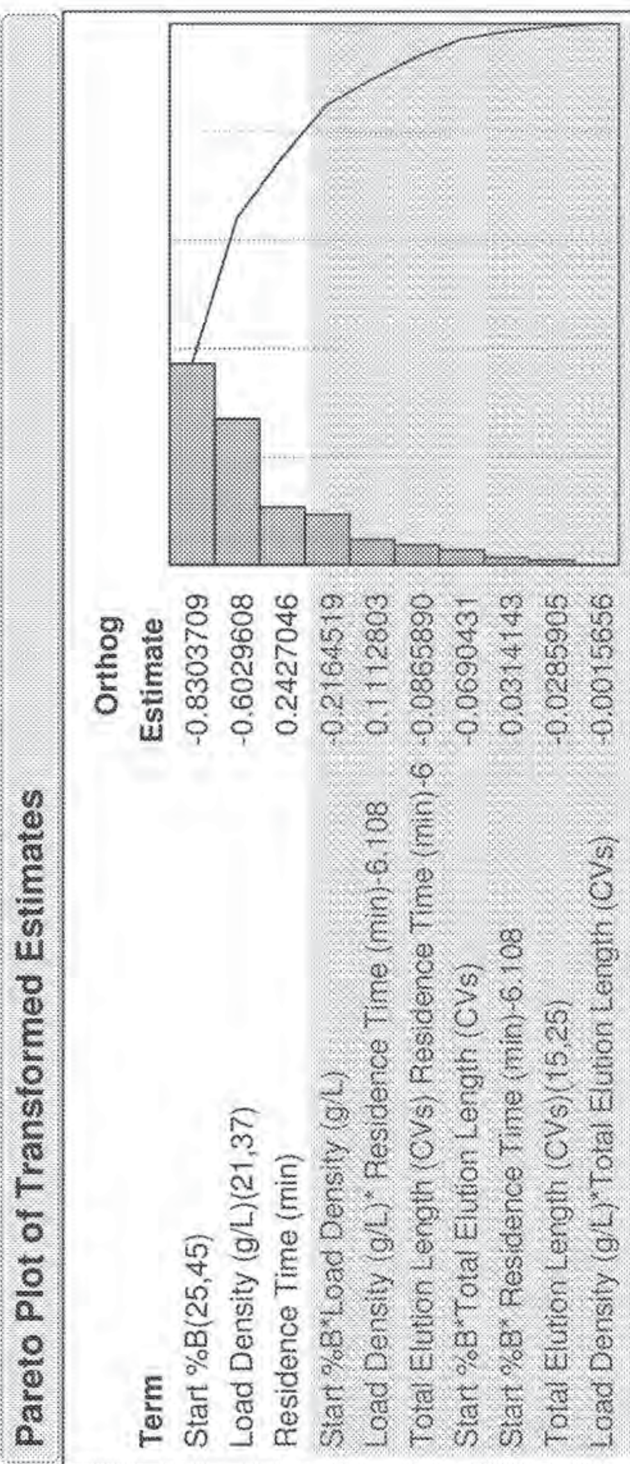
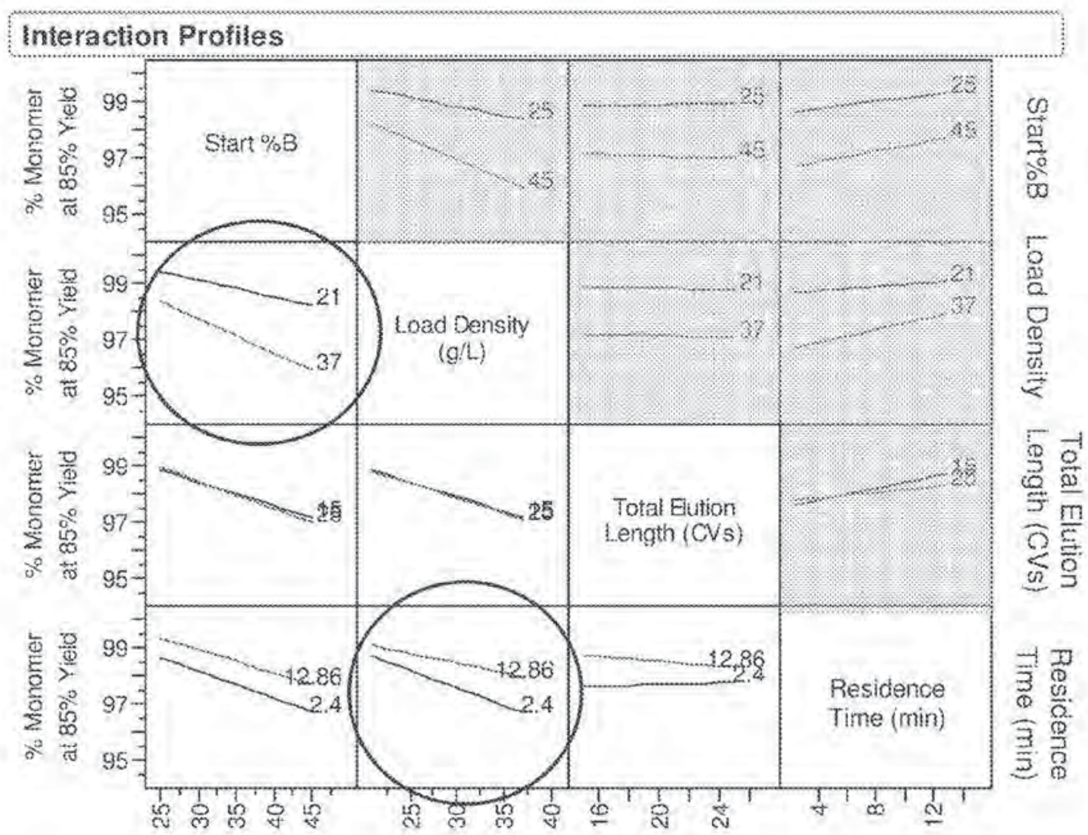


Figure 9

Figure 10



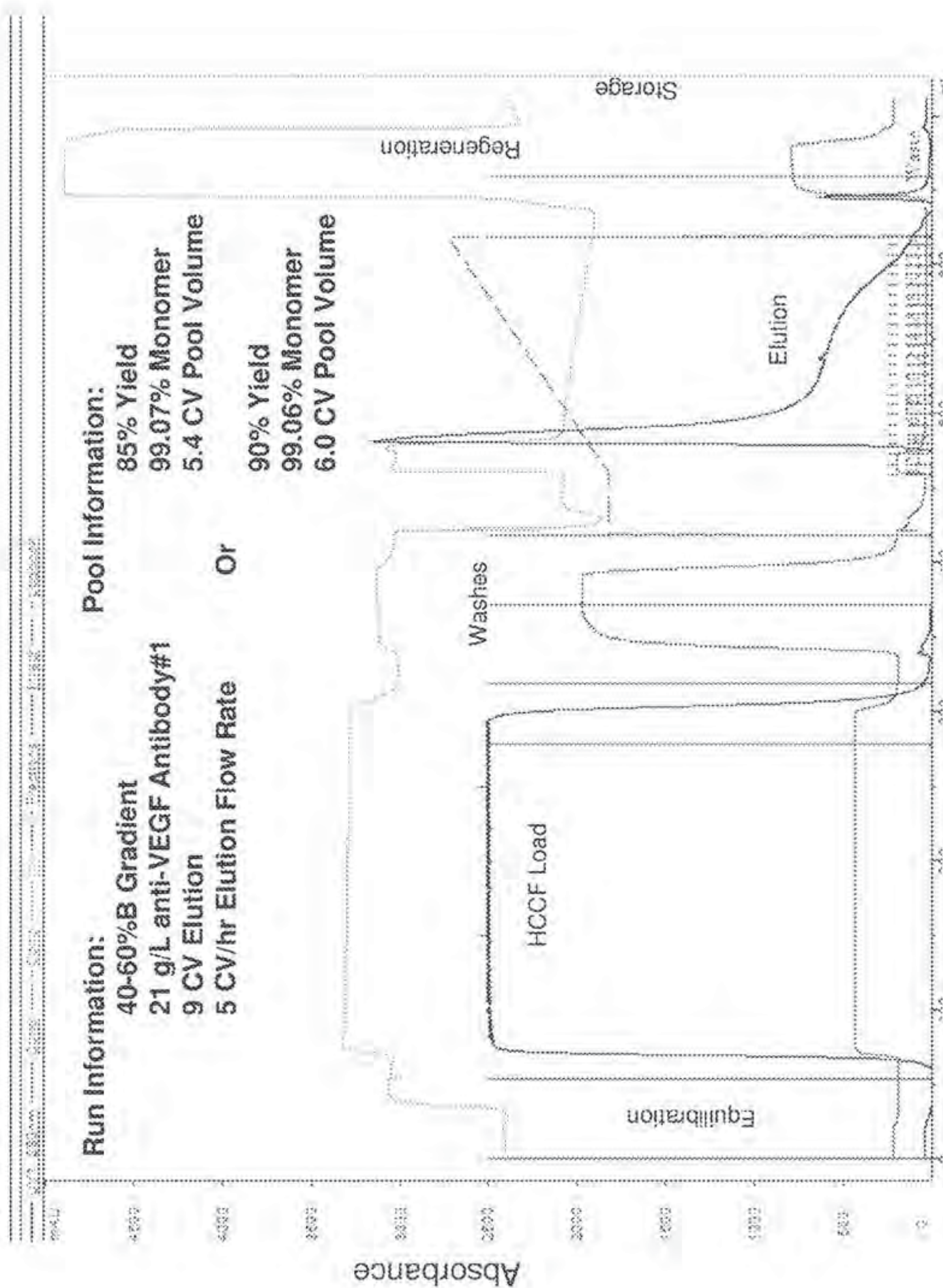


Figure 11
Run Information:
40-60%B Gradient
21 g/L anti-VEGF Antibody#1
9 CV Elution
5 CV/hr Elution Flow Rate
Pool Information:
85% Yield
99.07% Monomer
5.4 CV Pool Volume
Or
90% Yield
99.06% Monomer
6.0 CV Pool Volume
Absorbance
Equilibration
HCCF Load
Washes
Elution
Regeneration
Storage

Figure 12

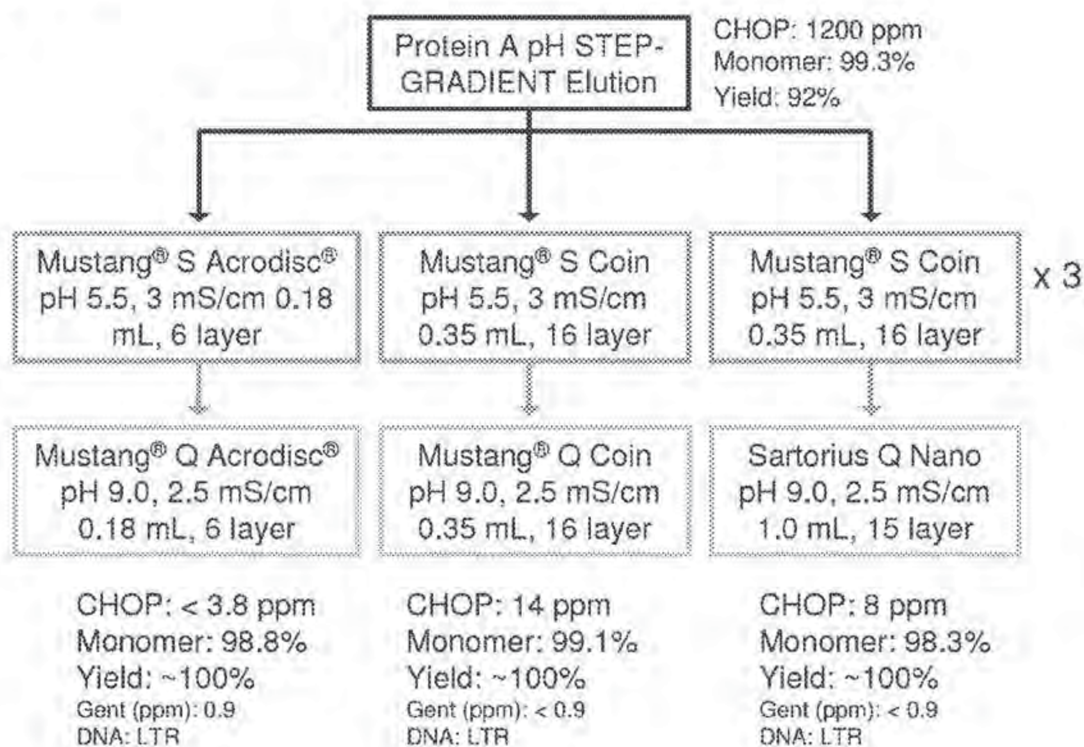


Figure 13

Anti-VEGF Antibody#1 Pilot Plant 4.1L Column SEC Results

% SEC Integration

100
80
60
40
20
0

Fraction

0 2 4 6 8 10 12 14 16 18 20

Pooled fractions 1-14:
86% Yield
99.3% Monomer

HMWS Dimer Monomer Fragment

Anti-VEGF Antibody#1 Small Scale 28mL Column SEC Results

% SEC Integration

100
80
60
40
20
0

Fraction

0 2 4 6 8 10 12 14 16 18 20

Pooled fractions 1-14:
88% Yield
99.2% Monomer

HMWS Dimer Monomer Fragment

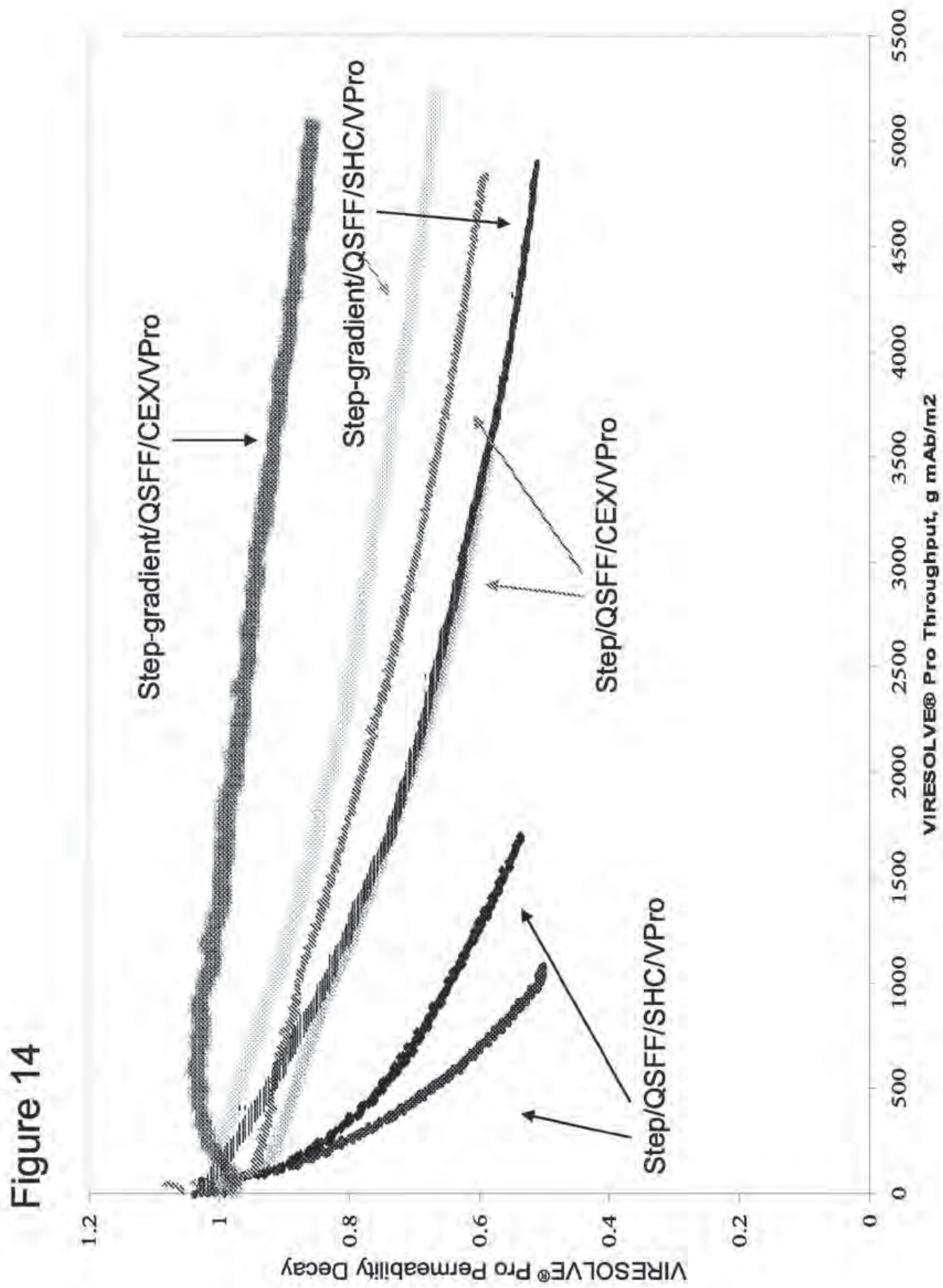


Figure 14
Step-gradient/QSFF/CEX/VPro
Step-gradient/QSFF/SHC/VPro
Step/QSFF/CEX/VPro
Step/QSFF/SHC/VPro
VIRESOLVE® Pro Permeability Decay
VIRESOLVE® Pro Throughput, g mAb/m2
1.2
1
0.8
0.6
0.4
0.2
0
500
1000
1500
2000
2500
3000
3500
4000
4500
5000
5500

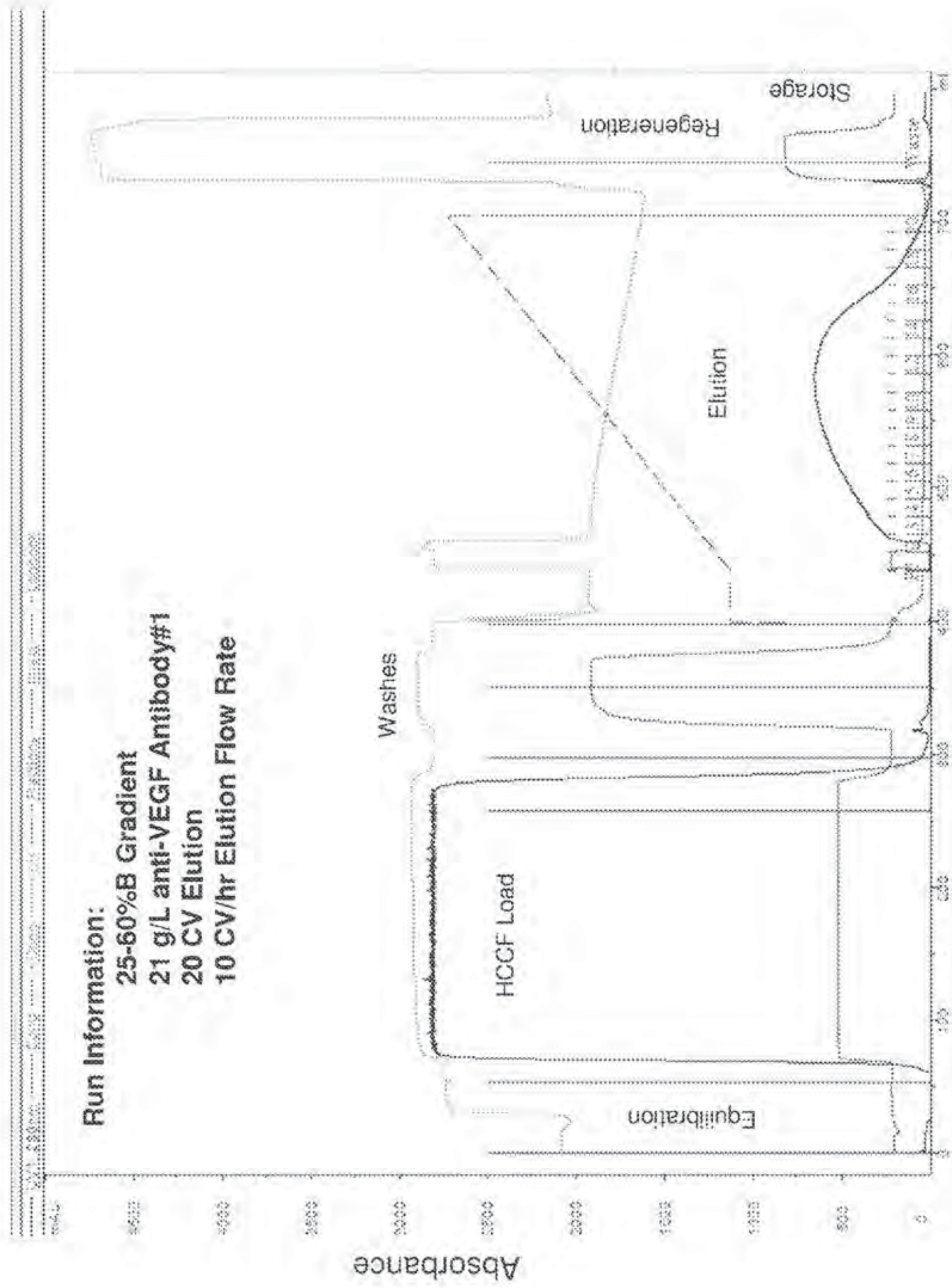


Figure 15

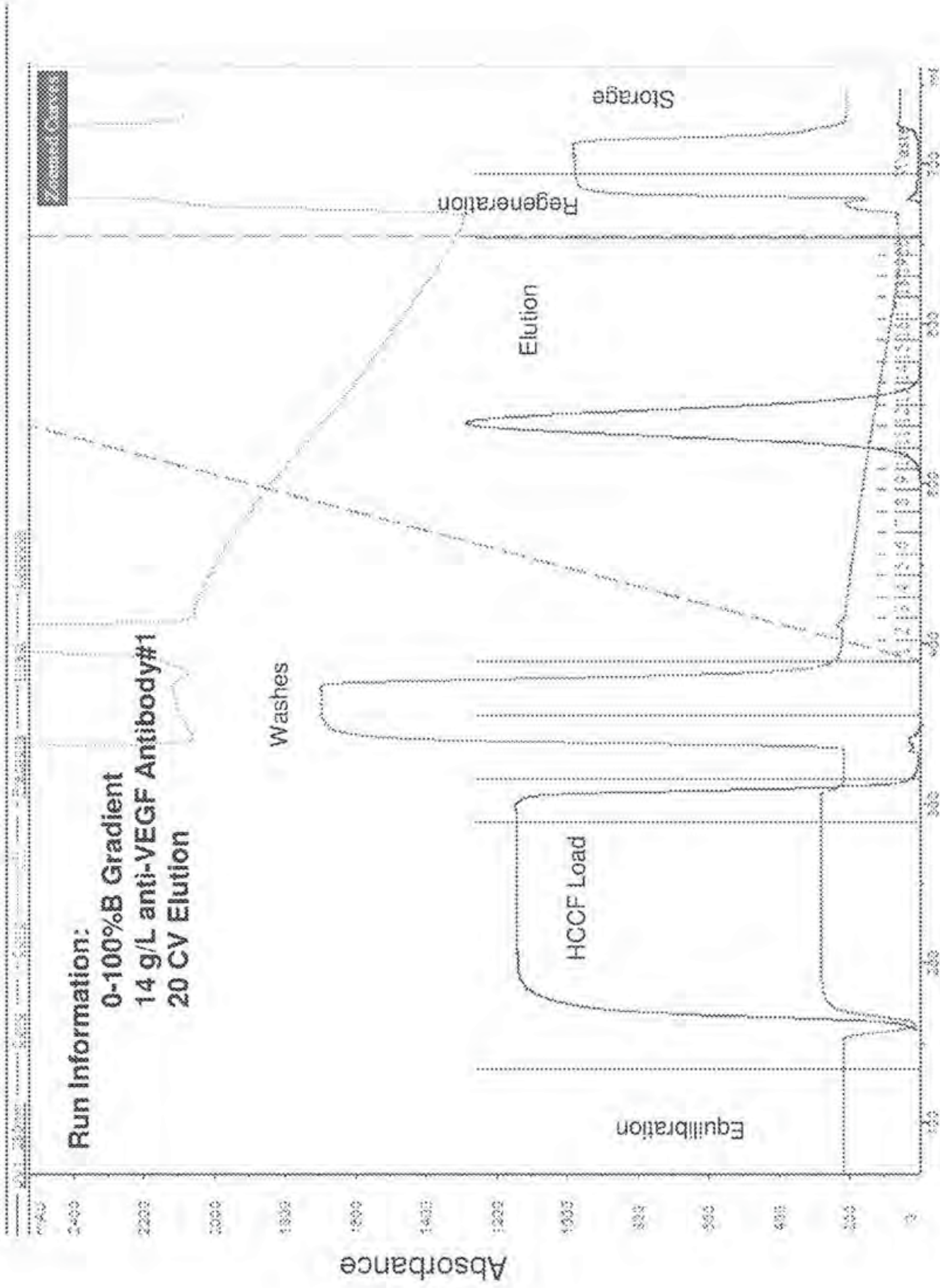


Figure 16
Run Information:
0-100%B Gradient
14 g/L anti-VEGF Antibody#1
20 CV Elution
Equilibration
HCCF Load
Washes
Elution
Regeneration
Storage
Absorbance

Figure 17

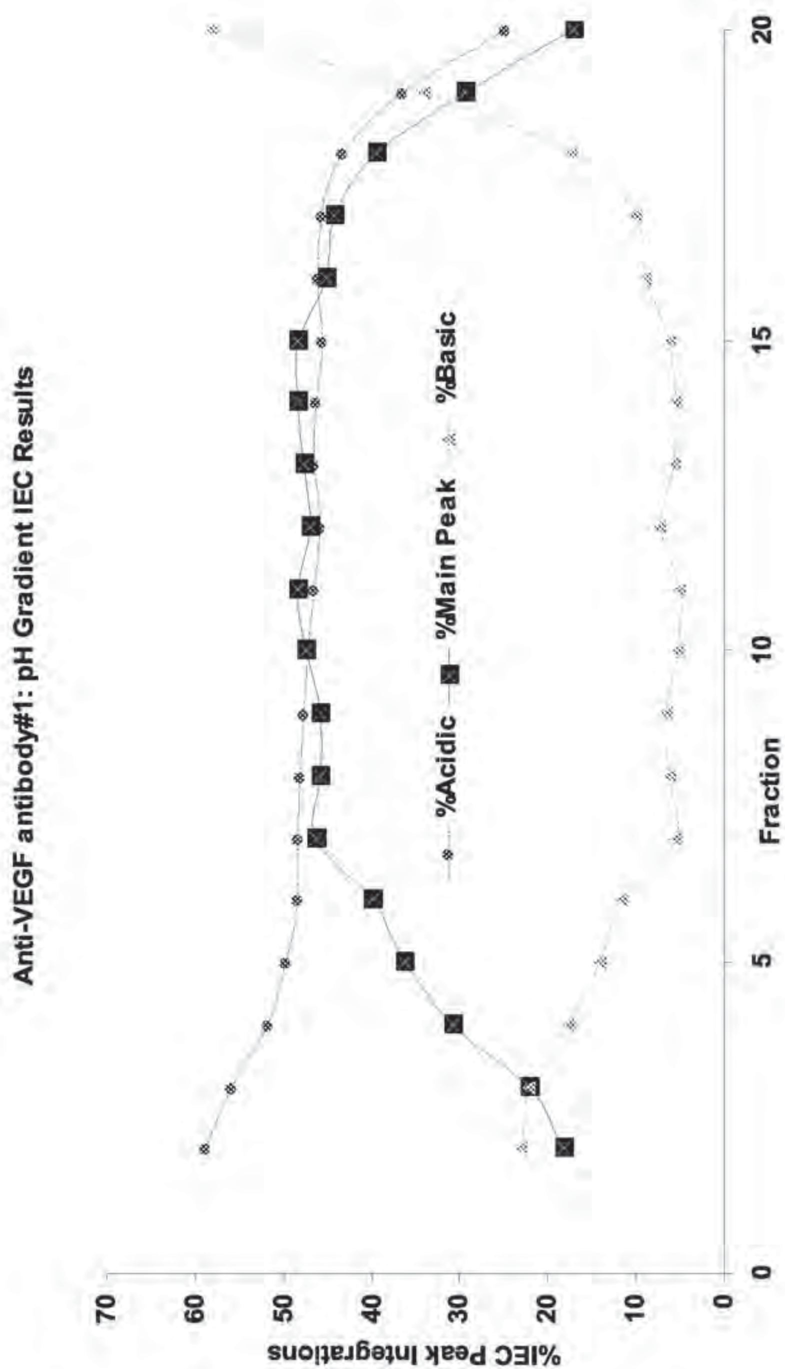


Figure 18

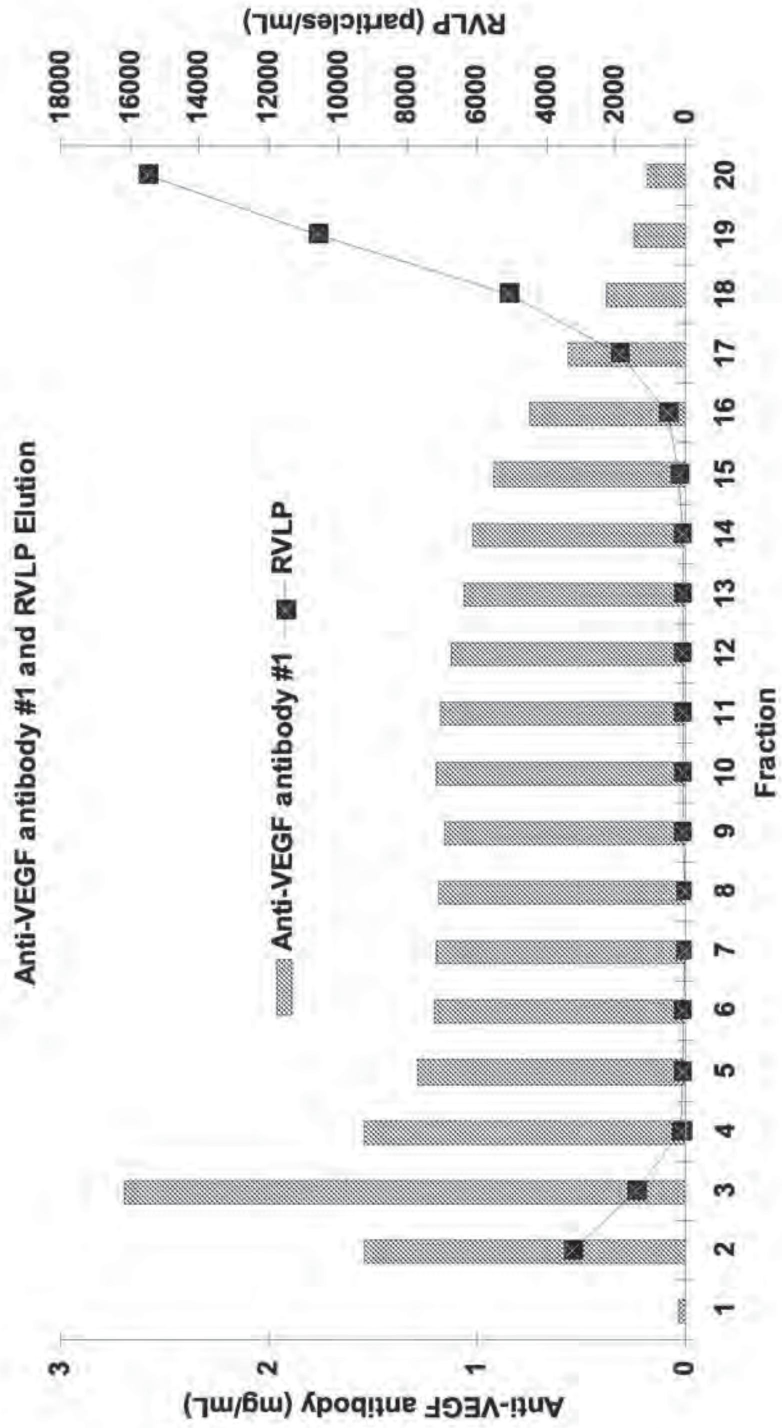


Figure 19

Anti-VEGF antibody #1 and RVLP Elution: LRV per fraction

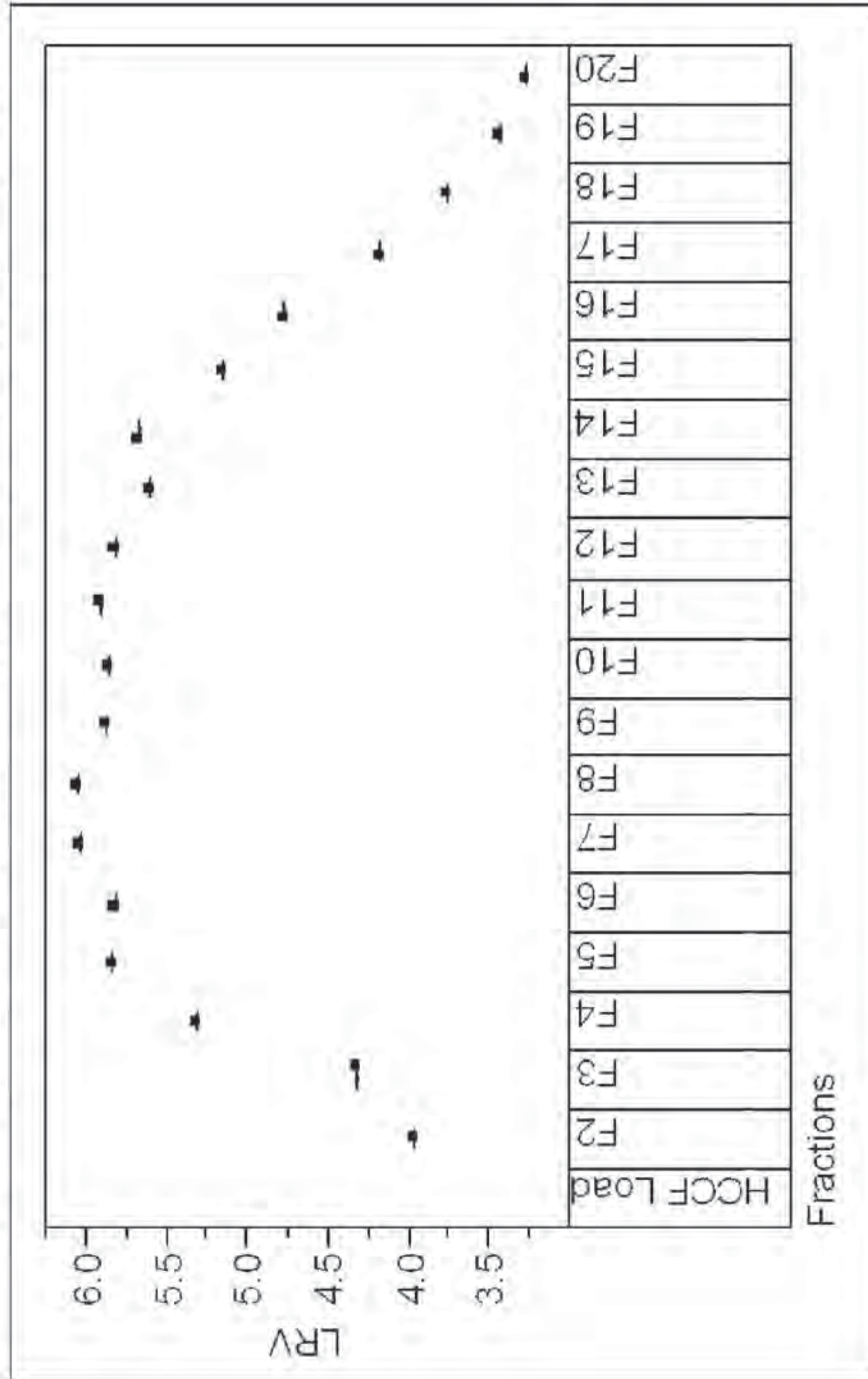
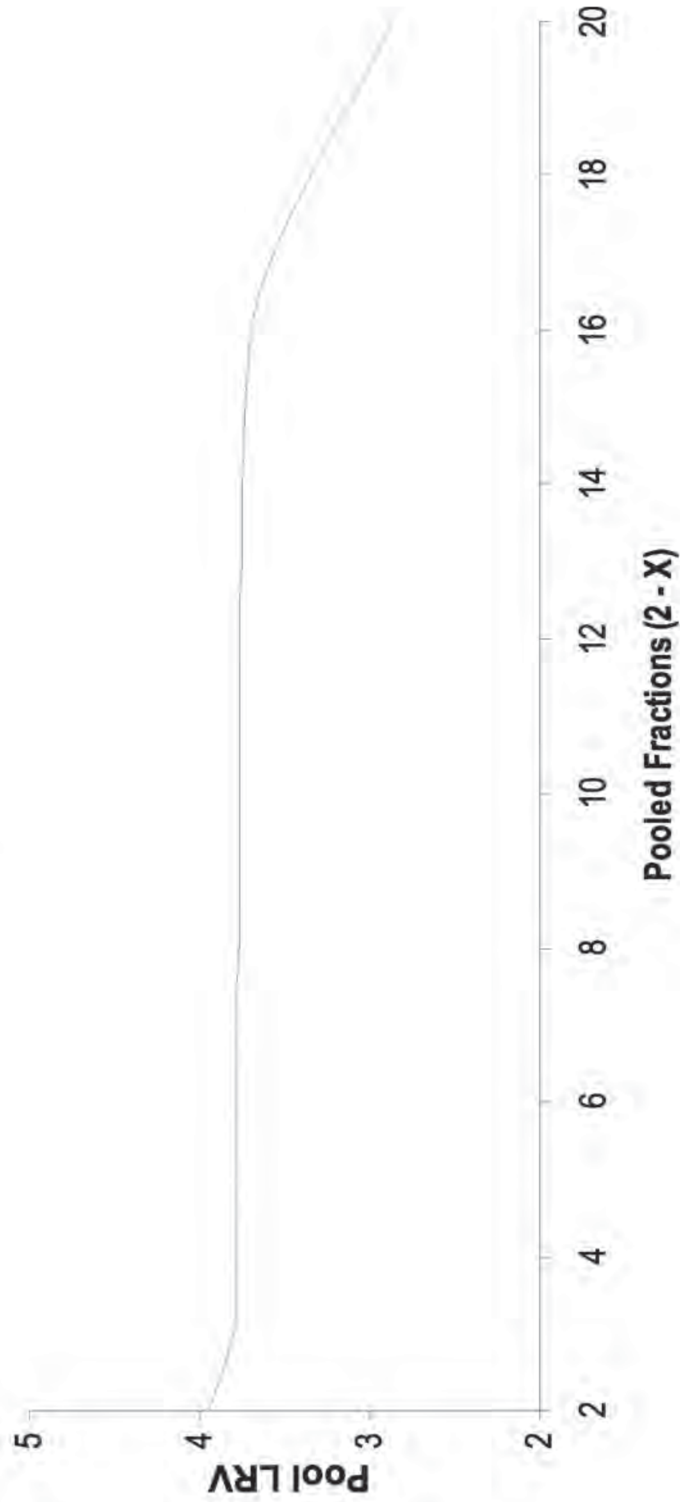


Figure 20

Mock Pool LRVs



ENHANCED PROTEIN PURIFICATION THROUGH A MODIFIED PROTEIN A ELUTION

CROSS-REFERENCE TO RELATED APPLICATIONS

This patent application is submitted under 35 U.S.C. §371 as a U.S. national stage application of International Patent Application No. PCT/US2010/047448, filed on Sep. 1, 2010 which claims priority to U.S. Provisional Patent Application No. 61/238,867, filed Sep. 1, 2009 and U.S. Provisional Patent Application No. 61/253,438 filed Oct. 20, 2009, the disclosure of each of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The field of this invention relates generally to methods for purifying a polypeptide comprising a C_H2/C_H3 region, comprising binding the polypeptide to Protein A and eluting with a pH gradient.

BACKGROUND OF THE INVENTION

The large-scale, economic purification of proteins is increasingly an important problem for the biotechnology industry. Generally, proteins are produced by cell culture, using either mammalian or bacterial cell lines engineered to produce the protein of interest by insertion of a recombinant plasmid containing the gene for that protein. Since the cell lines used are living organisms, they must be fed with a complex growth medium, containing sugars, amino acids, and growth factors, usually supplied from preparations of animal serum. Separation of the desired protein from the mixture of compounds fed to the cells and from the by-products of the cells themselves to a purity sufficient for use as a human therapeutic poses a formidable challenge.

Procedures for purification of proteins from cell debris initially depend on the site of expression of the protein. Some proteins can be caused to be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For the latter proteins, the first step of a purification process involves lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. The same problem arises, although on a smaller scale, with directly secreted proteins due to the natural death of cells and release of intracellular host cell proteins in the course of the protein production run.

Once a clarified solution containing the protein of interest has been obtained, its separation from the other proteins produced by the cell is usually attempted using a combination of different chromatography techniques. Affinity chromatography, which exploits a specific interaction between the protein to be purified and an immobilized capture agent, is commonly used for some proteins (e.g., proteins for use as a human therapeutic). Protein A is a useful adsorbent for affinity chromatography of proteins, such as antibodies, which contain an Fc region. Protein A is a 41 kD cell wall protein from *Staphylococcus aureas* which binds with a high affinity (about 10^{-8} M to human IgG) to the Fc region of antibodies. However, since proteins tend to aggregate or

become misfolded, the desired protein (i.e., monomer) is often co-purified with other impurities from these affinity columns, such as protein aggregates, by-products of the cells themselves (i.e., host cell impurities), or virus filter foulant.

Other techniques have been developed to further separate these impurities and mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size, such as ion exchange chromatography, hydrophobic interaction chromatography, or size exclusion chromatography. Several different chromatography resins or sorbents are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. The essence of each of these separation methods is that proteins can be caused either to move at different rates down a long solid phase (e.g., column), achieving a physical separation that increases as they pass further down the solid phase, or to adhere selectively to the separation medium, being then differentially eluted by different solvents. However, each of these methods requires additional buffers, resins or sorbents, and other resources for further purification, and this in turn results in longer processing time and higher cost. Thus, more efficient and economical methods for purifying protein monomers are needed.

Methods of purifying polypeptides from aggregates, multimers, and modified proteins using a protein A column and eluting with a pH gradient elution system was described in U.S. patent application Ser. No. 12/008,160.

All publications, patents, and patent applications cited herein are hereby incorporated by reference herein in their entirety for all purposes to the same extent as if each individual publication, patent, and patent application were specifically and individually indicated to be so incorporated by reference.

BRIEF SUMMARY OF THE INVENTION

The present invention provides methods for purifying a polypeptide comprising a C_H2/C_H3 region by binding the polypeptide to Protein A and eluting with a pH gradient starting at a low pH. These purification methods provide the advantages of achieving a better sequential separation of polypeptides or non-aggregates from various impurities, including host cell impurities, virus filter foulants, virus or virus-like particles, basic polypeptide variants, and polypeptide aggregates, and also a higher purity of the desirable polypeptide monomers in the purified fraction/pool. These methods can be achieved using various Protein A chromatography resins and chromatography sorbents. These methods can also be used at manufacturing scale and commercial process, and can facilitate the utilization of alternative downstream purification technologies other than column chromatography.

In one aspect, the invention provides a method for purifying a polypeptide comprising a C_H2/C_H3 region, comprising binding the polypeptide to Protein A and eluting with a pH gradient starting at or below 5.0.

In another aspect, the invention provides a method for purifying a polypeptide comprising a C_H2/C_H3 region, comprising the steps of: (a) binding the polypeptide to Protein A; and (b) eluting the polypeptide with a pH gradient starting at or below 5.0 using an elution buffer, wherein the elution buffer contains a high pH buffer and a low pH buffer and wherein the pH gradient is formed by adjusting a percentage of each pH buffer in the elution buffer.

In some embodiments, the pH gradient starts at about pH 4.2. In other embodiments, the pH gradient starts at about pH 4.3. In some embodiments, the pH gradient starts at

about 4.6. In some embodiments, the pH gradient ends at or above 3.0. In some embodiments, the pH gradient ends at about 3.7.

In some embodiments, the high pH buffer is at about pH 5.0 and wherein the low pH buffer is at about pH 2.7.

In some embodiments, the percentage of low pH buffer starts at about 35%. In some embodiments, the elution buffer containing the low pH buffer at about 35% comprises about 16.25 mM acetate and about 8.75 mM formate. In other embodiments, the percentage of low pH buffer starts at about 25%. In some embodiments, the elution buffer containing the low pH buffer the at about 25% comprises about 18.75 mM acetate and about 6.25 mM formate. In some embodiments, the percentage of low pH buffer starts at about 40%. In some embodiments, the elution buffer containing the low pH buffer at about 40% comprises about 15 mM acetate and about 10 mM formate.

In some embodiments, the polypeptide is loaded with a loading density starting at about 14 g/L. In some embodiments, the polypeptide is loaded with a loading density ranging from about 14 g/L to about 45 g/L.

In some embodiments, the Protein A is a Protein A column chromatography resin or a Protein A chromatography sorbent. In some embodiments, the Protein A chromatography sorbent is a membrane or a monolith.

In some embodiments, the Protein A is a Protein A column chromatography resin and wherein the polypeptide has an elution flow rate ranging from about 5 column volume/hour to about 25 column volume/hour.

In some embodiments, the Protein A is a Protein A column chromatography resin and wherein a purified fraction of the polypeptide contains about or fewer than about 12 Protein A column volumes.

In some embodiments, a host cell impurity is separated from the polypeptide. In some embodiments, the host cell impurity is Chinese Hamster Ovary Protein (CHOP).

In some embodiments, an aggregate is separated from the polypeptide. In other embodiments, a virus filter foulant is separated from the polypeptide.

In some embodiments, a virus particle or a virus-like particle is separated from the polypeptide. In other embodiments, a basic polypeptide variant is separated from the polypeptide.

In some embodiments, the C_H2/C_H3 region comprises a Fc region of an immunoglobulin.

In some embodiments, the polypeptide is an antibody. In some embodiments, the antibody is a monoclonal antibody, a polyclonal antibody, a multi-specific antibody, or an antibody fragment.

In other embodiments, the polypeptide is an immunoadhesion.

In some embodiments, the polypeptide has a purity of at least about 98% monomer. In other embodiments, the polypeptide has a purity of at least about 99% monomer.

In some embodiments, the ratio of a host cell impurity to the purified polypeptide is at least about 75% lower, about 80% lower, about 85% lower, about 90% lower, about 95% lower, about 96% lower, about 97% lower, about 98% lower, or about 99% lower than the ratio in unpurified polypeptide.

In some embodiments, a ratio of a host cell impurity to the purified polypeptide is at least about 20% lower than the ratio in a polypeptide purified by a step elution method, wherein the step elution method comprises binding the polypeptide to Protein A and eluting with a pH starting at or below 3.6. In some embodiments, a ratio of a host cell impurity to the purified polypeptide is at least about 60% lower than the ratio in a polypeptide purified by a step

elution method, wherein the step elution method comprises binding the polypeptide to Protein A and eluting with a pH starting at or below 3.6.

In some embodiments, the purified polypeptide has virus particle or virus-like particle count less than about 15000 particles/ml. In some embodiments, the purified polypeptide has virus particle or virus-like particle count less than about 12500 particles/ml, less than about 10000 particles/ml, less than about 7500 particles/ml, less than about 5000 particles/ml, less than about 2500 particles/ml, less than about 1500 particles/ml, less than about 1000 particles/ml, less than about 750 particles/ml, less than about 500 particles/ml, less than about 250 particles/ml, less than about 100 particles/ml, or less than about 50 particles/ml. In some embodiments, the virus-like particle is a retrovirus-like particle.

In some embodiments, the purified polypeptide has viral clearance of a virus or a virus-like particle at least about 4 LRV (log 10 reduction of virus). In some embodiments, the purified polypeptide has viral clearance of a virus or a virus-like particle ranging from about 4 LRV to about 8 LRV. In some embodiments, the purified polypeptide has viral clearance of a virus or a virus-like particle ranging from about 4 LRV to about 7 LRV. In some embodiments, the purified polypeptide has viral clearance of a virus or a virus-like particle at about 5 LRV, about 6 LRV, about 7 LRV, or about 8 LRV. In some embodiments, the virus-like particle is a retrovirus-like particle.

In some embodiments, the purified polypeptide is a polypeptide monomer.

In some embodiments, the Protein A is a modified or a non-modified Protein A ligand.

In some embodiments, the purification is a manufacturing scale process.

In some embodiments of any of the aspects of the invention, the purification method further comprises subjecting the polypeptide to a virus filtration step or an ion exchange chromatography step. In some embodiments, the ion exchange chromatography step runs after the purification step.

In some embodiments of any of the aspects of the invention, the purification method does not comprise a further purification step to remove an aggregate.

In some embodiments of any of the aspects of the invention, the purification method does not comprise a further purification step to remove a virus filter foulant.

In some embodiments of any of the aspects of the invention, the purification method does not comprise a further purification step to remove a basic polypeptide variant. In some embodiments of any of the aspects of the invention, the purification method does not comprise a further purification step to remove an acidic polypeptide variant.

In another aspect, the invention provides a polypeptide product purified by the methods described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the pH step-gradient chromatogram: x-axis is in mL from start of the Protein A run, and y-axis is absorbance (mAU). The distinctive shape of the step-gradient elution in the UV 280 trace is also shown—large peak is at the start of the elution and is then decreased to a stable height and tapers off at the lower pHs.

FIG. **2** shows the SEC (Size Exclusion Chromatography) results by fraction of anti-VEGF antibody #1. X-axis is retention time on SEC column (min), Y-axis is normalized UV absorbance (mAU). As fraction number increased (i.e., pH decreases as the gradient elution progresses), the HMWS

(High Molecular Weight Species) and dimer peaks (retention times of around 12.5 minutes and 13.5 minutes respectively) also increased while the monomer peak decreases (retention time of 16 minutes). The results from these curves were quantified by integration of all peaks (e.g., HMWS, dimer, and monomer), comparing the separate relative peak areas as percents (e.g., total area was set to 100%, a sample's SEC integration profile could give percents, such as "31% HMWS, 36% dimer, and 33% monomer").

FIG. **3** shows the SEC integration result graph for anti-VEGF antibody #1. This graph shows that the monomer levels were high for the first nine elution fractions with four fractions at 100%, and the dimer and HMWS levels peaked later in the elution. These assay results demonstrate that the pH step-gradient separates aggregates from the monomer of anti-VEGF antibody #1.

FIG. **4**A shows the SEC integration profile for multiple protein molecules (anti-CD20 antibody, anti-VEGF antibody #2, anti-MUC16, and anti-CD4 antibody). The pH step-gradient successfully separated the monomers from aggregates in anti-CD20 antibody, anti-VEGF antibody #2, anti-MUC16, and anti-CD4 antibody.

FIG. **4**B shows the SEC integration profile for an aglycosylated one-armed anti-Met antibody produced by a bacterial (*E. coli*) host cell fermentation. The pH step-gradient successfully separated monomers from aggregates in the aglycosylated one-armed anti-Met antibody.

FIG. **5** shows a side-by-side comparison between an anti-CD20 antibody standard step elution (control; eluting proteins at pH at or below 3.6 without the pH gradient) and pH step-gradient elution. The anti-CD20 antibody and CHOP elution graph on the left panel shows the CHOP levels per fraction in ppm (parts per million: unit used for standardizing measurement of impurities to amount of product). The anti-CD20 antibody and aggregate elution graph on the right panel shows the SEC peak integration values per fraction through the gradient elution. The vertical lines in both the left and right panels represent the mock pooling of the contained fractions that would generate a pH gradient elution pool with the characteristics shown in the table at the bottom of the slide.

FIG. **6** shows the CHOP separation for an anti-VEGF antibody #1. The CHOP levels per fraction are expressed in ppm or ng/mL.

FIG. **7** shows the CHOP separation for an anti-MUC16 antibody. The CHOP levels per fraction are expressed in ppm or ng/mL.

FIG. **8** is a MABSELECT™, MABSELECT SURE™, PROSEP® Va, PROSEP® Ultra Plus, and POROS® MAB-CAPTURE™ A Protein A resin chromatogram overlay.

FIG. **9** is a Pareto Plot showing that Start % B (the starting pH and the slope of the elution gradient) is the most influential parameter in determining aggregate separation efficiency, followed by load density and residence time.

FIG. **10** is an interaction profile for parameters of start % B, load density and start % B, total elution length, and residence time.

FIG. **11** is an exemplary manufacturing run for the pH step-gradient elution.

FIG. **12** shows the results of downstream ion exchange membrane study using two sizes of cation exchange membrane and three sizes of anion exchange membrane. The coin and nano units are both representative of manufacturing in the number of membrane layers, while the ACRODISC® units have a reduced number of layers, but are the typical bench scale models used.

FIG. **13** shows the SEC integration result comparison between a 4.1 L column (Pilot scale) and a 28 mL bench scale column run.

FIG. **14** is a VIRESOLVE® Pro permeability decay graph of anti-VEGF antibody #1 comparing the Protein A pH step-gradient versus the Protein A standard step in terms of facilitating a greater mass throughput over the VIRESOLVE® Pro parvovirus filter. There was about a six-fold increase in mass throughput over the VIRESOLVE® Pro using the protein A pH step-gradient.

FIG. **15** is a Protein A full pH gradient elution chromatogram showing the actual AKTA UNICORN™ chromatography software traces for the full pH gradient at a load density of 21 g/L. The initial tall UV 250 spike at the start of the gradient is missing, indicating that the pH at the start of the elution is higher than what is required to elute products from the Protein A column.

FIG. **16** is a pH 5.0-2.7 pH gradient trial (0-100% B) AKTA chromatogram, indicating that an anti-VEGF antibody #1 elutes as a discrete peak in the range of pH 4.6-3.6.

FIG. **17** is an ion exchange variant assay peak integration across Protein A pH step-gradient elution fractions, indicating the separation of basic polypeptide variant in the tail portion of the step-gradient elution.

FIG. **18** is RVLP (Retrovirus-like Particle) particle counts from QPCR (Quantitative Polymerase Chain Reaction) analysis per fraction graphed against anti-VEGF antibody #1 product elution. The majority of the RVLPs eluted late in the gradient where little product elution occurred.

FIG. **19** is LRV (log 10 reduction of virus) for each fraction in the Protein A pH step-gradient elution of anti-VEGF antibody #1.

FIG. **20** shows cumulative LRVs for mock pools of the fraction, showing that higher LRVs can be achieved in the Protein A pool if later fractions are omitted.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods for purifying a polypeptide comprising a C_H2/C_H3 region by binding the polypeptide to Protein A and eluting with a pH gradient starting at a low pH. The inventors have made the striking discovery that eluting polypeptides comprising a C_H2/C_H3 region from Protein A with a pH gradient at a low pH can provide a better sequential separation of polypeptides from various impurities, including host cell impurities, virus filter foulants, virus or virus-like particles, basic polypeptide variants, and/or polypeptide aggregates, and also achieve a higher purity or percentage of the desirable polypeptide monomers in the purified fraction/pool. Thus, the invention has significant advantages. The inventors have also discovered that these methods can be achieved using various Protein A chromatography resins and chromatography sorbents and that these methods can be used at manufacturing scale and commercial process and can facilitate the utilization of alternative downstream purification technologies other than column chromatography (e.g., membrane adsorbers).

Accordingly, in one aspect of the invention, provided is a method for purifying a polypeptide comprising a C_H2/C_H3 region, comprising binding the polypeptide to Protein A and eluting with a pH gradient starting at or below 5.0.

In another aspect of the invention, provided is a method for purifying a polypeptide comprising a C_H2/C_H3 region, comprising the steps of: (a) binding the polypeptide to Protein A; and (b) eluting the polypeptide with a pH gradient

starting at or below 5.0 using an elution buffer, wherein the elution buffer contains a high pH buffer and a low pH buffer and wherein the pH gradient is formed by adjusting a percentage of each pH buffer in the elution buffer.

In yet another aspect of the invention, provided is a polypeptide purified by the methods described herein.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; Oligonucleotide Synthesis (M. J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J. E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R. I. Freshney, ed., 1987); Introduction to Cell and Tissue Culture (J. P. Mather and P. E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J. B. Griffiths, and D. G. Newell, eds., 1993-1998) J. Wiley and Sons; Methods in Enzymology (Academic Press, Inc.); Handbook of Experimental Immunology (D. M. Weir and C. C. Blackwell, eds.); Gene Transfer Vectors for Mammalian Cells (J. M. Miller and M. P. Calos, eds., 1987); Current Protocols in Molecular Biology (F. M. Ausubel et al., eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Current Protocols in Immunology (J. E. Coligan et al., eds., 1991); Short Protocols in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C. A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: a practical approach (D. Catty., ed., IRL Press, 1988-1989); Monoclonal antibodies: a practical approach (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); Using antibodies: a laboratory manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995).

DEFINITIONS

It is understood that the polypeptide of interest herein is one which comprises a C_H2/C_H3 region and therefore is amenable to purification by Protein A. The term "C_H2/C_H3 region" when used herein refers to those amino acid residues in the Fc region of an immunoglobulin molecule which interact with Protein A. In some embodiments, the C_H2/C_H3 region comprises an intact C_H2 region followed by an intact C_H3 region, and most preferably comprises a Fc region of an immunoglobulin. Examples of C_H2/C_H3 region-containing proteins include antibodies, immunoadhesins and fusion proteins comprising a protein of interest fused to, or conjugated with, a C_H2/C_H3 region.

The terms "polypeptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art.

As used herein, the term "purified polypeptide" or "purified protein" is an eluted product from the Protein A affinity chromatography using the pH gradient methods as described herein. Purified polypeptides/proteins preferably contain mostly polypeptide monomers.

As used herein, the term "unpurified polypeptide," "unpurified protein," or "protein load" is a polypeptide or protein in the loading material or starting material prior to the Protein A affinity chromatography purification step.

As used herein, the term "impurity" or "impurities" is a material that is different from the desired polypeptide monomer product. The impurities include, but are not limited to, a polypeptide variant (e.g., acidic or basic polypeptide variant), polypeptide fragment, aggregate or derivative of the desired polypeptide monomer, another polypeptide, lipid, nucleic acid, endotoxin, host cell impurity, or virus filter foulant.

As used herein, the term "monomer(s)" refers to a single unit of a polypeptide comprising a C_H2/C_H3 region. For example, in the case of an antibody, a monomer consists of two heavy chains and two light chains; in the case of a one-armed antibody, a monomer consists of one heavy chain and one light chain.

As used herein, the term "basic polypeptide variant" or 'basic variant" refers to a variant of a polypeptide of interest which is more basic (e.g., as determined by cation exchange chromatography) than the polypeptide of interest.

As used herein, the term "acidic polypeptide variant" or 'acidic variant" refers to a variant of a polypeptide of interest which is more acidic (e.g., as determined by cation exchange chromatorgraphy) than the polypeptide of interest.

As used herein, the term "aggregate(s)" refers to any multimers of a polypeptide or a polypeptide fragment comprising a C_H2/C_H3 region. For example, an aggregate can be a dimer, trimer, tetramer, or a multimer greater than a tetramer, etc.

As used herein, the term "host cell impurity" refers to any proteinaceous contaminant or by-product introduced by the host cell line, cell cultured fluid, or cell culture. Examples include, but are not limited to, Chinese Hamster Ovary Protein (CHOP), *E. coli*. Protein, yeast protein, simian COS protein, or myeloma cell protein (e.g., NSO protein (mouse plastocytoma cells derived from a BALB/c mouse)).

As used herein, the term "virus filter foulant" refers to any large molecular weight particle or high molecular weight species (HMWS) with a hydrodynamic diameter similar to or greater than the pore size distribution of the parvovirus filter. Virus filter foulants include, but are not limited to, soluble high molecular weight polypeptide aggregates, and soluble and/or insoluble aggregates of host cell impurities (e.g., CHOP).

A "host cell" includes an individual cell or cell culture that can be or has been a recipient for vector(s) for incorporation of polynucleotide inserts to produce polypeptides. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in genomic DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation.

The "solid phase," as used herein, refers to a non-aqueous matrix to which the Protein A can adhere.

A "buffer" is a buffered solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in *Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., Ed. Calbiochem Corporation (1975).

The "equilibration buffer" herein is that used to prepare the solid phase (with immobilized Protein A) for loading the protein of interest.

The "wash buffer" is used herein to refer to the buffer that is passed over the solid phase (with immobilized Protein A) following loading and prior to elution of the protein of interest.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they retain, or are modified to comprise, a C_H2/C_H3 region as herein defined.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; single-chain antibody molecules; diabodies; linear antibodies; and multispecific antibodies formed from antibody fragments. As used herein, the antibody fragment comprises a C_H2/C_H3 region.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al, *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (i.e. residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Proteins of Immunological Interest,* 5 th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (i.e. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the "binding domain" of a heterologous "adhesin" protein (e.g. a receptor, ligand or enzyme) with the effector functions of an immunoglobulin constant domain. Structurally, the immunoadhesins comprise a fusion of the adhesin amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site (antigen combining site) of an antibody (i.e. is "heterologous") and an immunoglobulin constant domain sequence. The immunoglobulin constant domain sequence in the immunoadhesin is preferably derived from γ1, γ2, or γ4 heavy chains since immunoadhesins comprising these regions can be purified by Protein A chromatography (Lindmark et al., *J. Immunol. Meth.* 62:1-13 (1983)).

The term "ligand binding domain" as used herein refers to any native cell-surface receptor or any region or derivative thereof retaining at least a qualitative ligand binding of a corresponding native receptor. In a specific embodiment, the receptor is from a cell-surface polypeptide having an extracellular domain which is homologous to a member of the immunoglobulin supergenefamily. Other receptors, which are not members of the immunoglobulin supergenefamily but are nonetheless specifically covered by this definition, are receptors for cytokines, and in particular receptors with tyrosine kinase activity (receptor tyrosine kinases), members of the hematopoietin and nerve growth factor receptor superfamilies, and cell adhesion molecules, e.g. (E-, L- and P-) selectins.

The term "receptor binding domain" is used to designate any native ligand for a receptor, including cell adhesion molecules, or any region or derivative of such native ligand retaining at least a qualitative receptor binding ability of a

corresponding native ligand. This definition, among others, specifically includes binding sequences from ligands for the above-mentioned receptors.

An "antibody-immunoadhesin chimera" comprises a molecule which combines at least one binding domain of an antibody (as herein defined) with at least one immunoadhesin (as defined in this application). Exemplary antibody-immunoadhesin chimeras are the bispecific CD4-IgG chimeras described in Berg et al., *PNAS* (USA) 88:4723-4727 (1991) and Chamow et al., *J. Immunol.* 153:4268 (1994).

For use herein, unless clearly indicated otherwise, use of the terms "a", "an," and the like refers to one or more.

Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X." Numeric ranges are inclusive of the numbers defining the range.

It is understood that wherever embodiments are described herein with the language "comprising," otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided.

Purification of Polypeptides

The process herein involves purifying a C_H2/C_H3 region-containing polypeptide from one or more impurities by Protein A affinity chromatography using a pH gradient starting at a low pH. In one aspect, polypeptide comprising a C_H2/C_H3 region can be purified by a method comprising binding the polypeptide to Protein A and eluting with a pH gradient starting at or below 5.0.

In another aspect, polypeptide comprising a C_H2/C_H3 region can also be purified by a method comprising the steps of: (a) binding the polypeptide to Protein A; and (b) eluting the polypeptide with a pH gradient starting at or below 5.0 using an elution buffer, wherein the elution buffer contains a high pH buffer and a low pH buffer and wherein the pH gradient is formed by adjusting a percentage of each pH buffer in the elution buffer.

Protein A can be a modified or a non-modified Protein A ligand. As used herein, "Protein A ligand" encompasses native Protein A, Protein A produced synthetically (e.g. by peptide synthesis or by recombinant techniques), and variants thereof which retain the ability to bind proteins which have a C_H2/C_H3 region. A modified Protein A ligand can be chemically engineered to be stable in high pH solutions for short amounts of time (e.g., MABSELECT SURE™ (GE Healthcare (Piscataway, N.J.)), POROS® MABCAPTURE™ A (Applied Biosystems (Foster City, Calif.)). The term "non-modified Protein A ligand," as used herein, encompasses Protein A ligand that is similar to Protein A recovered from a native source. Non-modified Protein A ligand, for example, MABSELECT™, PROSEP™ Va, PROSEP™ Ultra Plus, can be purchased commercially from GE Healthcare (Piscataway, N.J.) or Millipore (Billerica, Mass.).

The Protein A can be immobilized on a solid phase. The solid phase may be a purification column, a discontinuous phase or discrete particles, a membrane, or filter. Examples of materials for forming the solid phase include polysaccharides (such as agarose and cellulose) and other mechanically stable matrices such as silica (e.g., controlled pore glass), poly(styrenedivinyl)benzene, polyacrylamide, ceramic particles, and derivatives of any of the above.

Protein A immobilized on a solid phase is used to purify the C_H2/C_H3 region-containing polypeptides. In some embodiments, the solid phase is a Protein A column resin comprising a glass bead-based resin, silica-based resin, or agarose-based resin for immobilizing the Protein A. For

example, the solid phase is a controlled pore glass column or a silicic acid column. Sometimes, the column has been coated with a reagent, such as glycerol, in an attempt to prevent nonspecific adherence to the column. The PROSEP™ A column is an example of a Protein A controlled pore glass column which is coated with glycerol. In other embodiments, the solid phase is a Protein A chromatography sorbent for immobilizing the Protein A. Protein A chromatography sorbents include, but are not limited to, membranes (e.g. Sartorius (Goettingen, Germany), SARTOBIND™ Protein A membrane) or monoliths (e.g., BIA Separations (Villach, Austria), CIM® Protein A HLD monoliths).

The solid phase for the Protein A chromatography can be equilibrated with an equilibration buffer, and the unpurified polypeptides comprising various impurities (e.g., harvested cell culture fluid) can then be loaded onto the equilibrated solid phase. The polypeptide can be loaded with a loading buffer. Conveniently, the equilibration buffer for equilibrating the solid phase can be the same as the loading buffer, but this is not required. As the polypeptides flow through the solid phase, the polypeptides and various impurities are adsorbed to the immobilized protein A. The wash buffers can be used to remove some impurities, such as host cell impurities, but not polypeptides of interest.

The equilibration buffer is preferably isotonic and commonly has a pH in the range from about 6 to about 8. For example, an equilibration buffer can have 25 mM Tris, 25 mM NaCl, 5 mM EDTA, and pH 7.1.

The "loading buffer" refers to a buffer that is used to load the mixture of the C_H2/C_H3 region-containing protein and contaminants onto the solid phase to which the Protein A is immobilized. Often, the equilibration and loading buffers are the same.

The wash buffer may serve to elute cell line impurity or other various impurities. The conductivity and/or pH of the wash buffer is/are such that the impurities are eluted from the Protein A chromatography, but not any significant amounts of the polypeptide of interest.

The polypeptide bound to Protein A can be eluted with a pH gradient using a single elution buffer or a combination of elution buffers.

The "elution buffer" is used to elute the C_H2/C_H3 region-containing polypeptide from the immobilized Protein A. As used herein, the elution buffer contains a high pH buffer and a low pH buffer and thereby forms a pH gradient by adjusting a percentage of the high pH buffer and the low pH buffer in the elution buffer. In some embodiments, the elution buffer has a pH in the range from about 3 to about 5. The pH values as used herein are measured without the presence of polypeptides. Examples of pH buffers that control the pH within this range include, but are not limited to, phosphate, acetate, citrate, formic acid, and ammonium buffers, as well as combinations of these. The preferred such buffers are acetate, and formic acid buffers.

In some embodiments, the pH gradient starts at about 5.0. In some embodiments, the pH gradient starts at below 5.0. In some embodiment, the pH gradient starts ranging from about 5.0 to about 4.0. In some embodiments, the pH gradient starts at about 4.9, about 4.8, about 4.7, about 4.6, about 4.5, about 4.4, about 4.3, about 4.2, about 4.1, or about 4.0. In some embodiments, the pH gradient starts at about 4.98, about 4.96, about 4.94, about 4.92, about 4.90, about 4.88, about 4.86, about 4.84, about 4.82, about 4.80, about 4.78, about 4.76, about 4.74, about 4.72, about 4.70, about 4.68, about 4.66, about 4.64, about 4.62, about 4.60, about 4.58, about 4.56, about 4.54, about 4.52, about 4.50, about

4.48, about 4.46, about 4.44, about 4.42, about 4.40, about 4.38, about 4.36, about 4.34, about 4.32, about 4.30, about 4.28, about 4.24, about 4.22, about 4.20, about 4.18, about 4.16, about 4.14, about 4.12, about 4.10, about 4.08, about 4.06, about 4.04, or about 4.02.

In some embodiments, the pH gradient ends at about 3.0. In some embodiments, the pH gradient ends at above 3.0. In some embodiments, the pH gradient ends ranging from about 3.0 to about 4.0. In some embodiments, the pH gradient ends at about 3.1, about 3.2, about 3.3, about 3.4, about 3.5, about 3.6, about 3.7, about 3.8, or about 3.9. In some embodiments, the pH gradient ends at about 3.12, about 3.14, about 3.16, about 3.18, about 3.20, about 3.22, about 3.24, about 3.26, about 3.28, about 3.30, about 3.32, about 3.34, about 3.36, about 3.38, about 3.40, about 3.42, about 3.44, about 3.46, about 3.48, about 3.50, about 3.52, about 3.54, about 3.56, about 3.58, about 3.60, about 3.61, about 3.62, about 3.63, about 3.64, about 3.65, about 3.66, about 3.67, about 3.68, about 3.69, about 3.70, about 3.71, about 3.72, about 3.73, about 3.74, about 3.75, about 3.76, about 3.77, about 3.78, about 3.79, about 3.80, about 3.82, about 3.84, about 3.86, about 3.88, about 3.9, about 3.92, about 3.94, about 3.96, or about 3.98.

In some embodiments, the pH gradient starts at about pH 4.2 and ends at about pH 3.7. In some embodiments, the pH gradient starts at about pH 4.24 and ends at about pH 3.69. For example, anti-VEGF antibodies, anti-CD20 antibodies, anti-MUC16 antibodies, anti-CD4 antibodies, and one armed anti-Met antibodies can be purified using the pH gradient starting at about pH 4.24 and ends at about pH 3.69.

In other embodiments, the pH gradient starts at about pH 4.3 and ends at about pH 3.7. In some embodiments, the pH gradient (i.e., pH step-gradient) starts at about pH 4.34 and ends at about pH 3.69. For example, anti-VEGF antibodies, anti-CD20 antibodies, anti-MUC16 antibodies, anti-CD4 antibodies, and one armed anti-Met antibodies can be purified using the pH gradient starting at about pH 4.34 and ends at about pH 3.69.

In some embodiments, the pH gradient starts at about pH 4.6 and ends at about pH 3.7. In some embodiments, the pH gradient (i.e., pH full-gradient) starts at about pH 4.58 and ends at about pH 3.69. For example, anti-VEGF antibodies, anti-CD20 antibodies, anti-MUC16 antibodies, anti-CD4 antibodies, and one armed anti-Met antibodies can be purified using the pH gradient starting at about pH 4.58 and ends at about pH 3.69.

The elution buffer contains a high pH buffer and a low pH buffer and the pH gradient is formed by adjusting a percentage of each pH buffer in the elution buffer. In some embodiments, the high pH buffer is at about pH 5.0 and the low pH buffer is at about 2.7. For example, the high pH buffer can be 25 mM acetate and pH 5.0, and the low pH buffer can be 25 mM formic acid and pH 2.7.

Adjusting the starting percentage of the low pH buffer can optimize and maximize the purity of the purified polypeptide, and also the sequential separation of the impurities, including aggregates, cell line impurities, basic polypeptide variant, virus particle, virus-like particle, and virus filter foulants, from the polypeptide monomers. The percentage of low pH buffer in the elution buffer can start at about 25%, about 30%, about 35%, about 40%, or about 45%.

In some embodiments, the percentage of low pH buffer in the elution buffer can start at about 25%. In some embodiments, the elution buffer containing the low pH buffer at about 25% comprises about 19 mM acetate, about 6 mM formate, and about 1140 buffer conductivity at pH 4.5-4.6. For example, the elution buffer containing the low pH buffer at about 25% comprises 18.75 mM acetate, 6.25 mM formate, 1141 uS/cm buffer conductivity, at pH 4.58.

In some embodiments, the percentage of low pH buffer in the elution buffer can start at about 35%. In some embodiments, the elution buffer containing the low pH buffer at about 35% comprises about 16 mM acetate, about 9 mM formate, and about 1040 buffer conductivity at pH 4.3-4.4. For example, elution buffer containing the low pH buffer at about 35% comprises 16.25 mM acetate, 8.75 mM formate, 1039 uS/cm buffer conductivity, at pH 4.34.

In some embodiments, the percentage of low pH buffer in the elution buffer can start at about 40%. In some embodiments, the elution buffer containing the low pH buffer at about 40% comprises about 15 mM acetate, about 10 mM formate, and about 974 buffer conductivity at pH 4.2-4.3. For example, the elution buffer containing the low pH buffer at about 40% comprises 15 mM acetate, 10 mM formate, 974 uS/cm buffer conductivity, at pH 4.24.

In some embodiments, the percentage of low pH buffer in the elution buffer can end at about 60%. In some embodiments, the elution buffer containing the low pH buffer at about 60% comprises about 10 mM acetate, about 15 mM formate, and about 763 buffer conductivity at pH 3.6-3.7. For example, the low pH buffer at the end of the pH gradient can be 10 mM acetate, 15 mM formate, 763 uS/cm buffer conductivity, at pH 3.69.

In some embodiments, the elution buffer has a buffer conductivity ranging from about 1200 uS/cm to about 500 uS/cm. In some embodiments, the elution buffer has a buffer conductivity ranging from about 1150 uS/cm to about 700 uS/cm. In some embodiments, the elution buffer has a buffer conductivity of about 1145 uS/cm, about 1141 uS/cm, about 1130 uS/cm, about 1120 uS/cm, about 1110 uS/cm, about 1000 uS/cm, about 1039 uS/cm, about 1000 uS/cm, about 974 uS/cm, about 900 uS/cm, about 800 uS/cm, about 763 uS/cm, or about 700 uS/cm.

In some embodiments, the composition of the elution buffer is about 9-20 mM acetate and 5-15 mM formate. In some embodiments, the composition of the elution is about 10-19 mM acetate and 6-16 mM formate.

Adjusting the loading density of the polypeptide can also optimize and maximize the purity of the purified polypeptide and the separation of the impurities, including aggregates, cell line impurities, basic polypeptide variant, virus particle, virus-like particle, and virus filter foulants, from the polypeptide monomers.

The term "load density" or "loading density" is the density of the purified polypeptide (g) per liter of chromatography resin or the density of the purified polypeptide per liter of membrane/filter volume (L). The loading density is measured in g/L.

In some embodiments, the polypeptide is loaded with a loading density starting at or above 14 g/L. In some embodiments, the polypeptide is loaded with a loading density ranging from about 14 g/L to about 45 g/L or from about 14 g/L to about 70 g/L. In some embodiments, the polypeptide is loaded with a loading density at about 15 g/L, about 17 g/L, about 19 g/L, about 21 g/L, about 23 g/L, about 25 g/L, about 26 g/L, about 27 g/L, about 28 g/L, about 29 g/L, about 31 g/L, about 33 g/L, about 35 g/L, about 37 g/L, about 39 g/L, about 41 g/L, about 43 g/L, about 45 g/L, about 50 g/L, about 55 g/L, about 60 g/L, about 65 g/L, or about 70 g/L.

Adjusting the polypeptide elution residence time (or elution flow rate) can also optimize and maximize polypeptide purity and the sequential separation of impurities from the polypeptide monomers. At increased loading density, the

polypeptide elution residence time plays a much larger role in the ability of the pH gradient to fractionate aggregates efficiently. In some embodiments, the polypeptide has an elution flow rate ranging from about 5 column volume/hour to about 35 column volume/hour. In some embodiments, the polypeptide has an elution flow rate ranging from about 5 column volume/hour to about 25 column volume/hour. In some embodiments, the polypeptide has an elution flow rate of about 5 column volume/hour, about 7.5 column volume/hour, about 10 column volume/hour, about 12.5 column volume/hour, about 15 column volume/hour, about 17.5 column volume/hour, about 20 column volume/hour, about 22.5 column volume/hour, about 25 column volume/hour, about 27.5 column volume/hour, about 30 column volume/hour, about 32.5 column volume/hour, or about 35 column volume/hour.

Polypeptides purified using the methods described herein have a yield of at least about any of 75% unpurified polypeptide, 80% unpurified polypeptide, 85% unpurified polypeptide, 90% unpurified polypeptide, 95% unpurified polypeptide, 96% unpurified polypeptide, 97% unpurified polypeptide, 98% unpurified polypeptide, or 99% unpurified polypeptide.

Yield is the total amount of purified polypeptide collected in comparison to the unpurified polypeptide prior to the Protein A affinity chromatography purification as described herein, usually expressed as a percentage of the unpurified polypeptide.

In some embodiments, the ratio of a host cell impurity to the purified polypeptide is at least about 75% lower, about 80% lower, about 85% lower, about 90% lower, about 95% lower, about 96% lower, about 97% lower, about 98% lower, or about 99% lower than the ratio in unpurified polypeptide.

In some embodiments, the ratio of a host cell impurity to the purified polypeptide is at least about 20% lower, about 30% lower, about 40% lower, about 50% lower, about 60% lower, or about 70% lower than the ratio in the polypeptide purified using a pH purification step(s) other than those of the instant invention. For example, in a conventional or typical step Protein A elution method, the polypeptide is purified by binding the polypeptide to Protein A and eluting the polypeptide at or below pH 3.6 without the pH gradient. Accordingly, in some embodiments, the ratio of a host cell impurity to the purified polypeptide is at least about 20% lower, about 30% lower, about 40% lower, about 50% lower, about 60% lower, or about 70% lower than the ratio in a polypeptide purified by a step elution method, wherein the step elution method comprises binding the polypeptide to Protein A and eluting with a pH starting at or below 3.6.

In some embodiments, the ratio of a virus filter foulant to the purified polypeptide is at least about 75% lower, at least about 80% lower, about 85% lower, about 90% lower, about 95% lower, about 96% lower, about 97% lower, about 98% lower, or about 99% lower than the ratio in unpurified polypeptide.

In some embodiments, the ratio of a virus filter foulant to the purified polypeptide is at least about 20% lower, about 30% lower, about 40% lower, about 50% lower, about 60% lower, or about 70% lower than the ratio in the polypeptide purified using a pH purification step(s) other than those of the instant invention. For example, in a conventional or typical step Protein A elution method, the polypeptide is purified by binding the polypeptide to Protein A and eluting the polypeptide at or below pH 3.6 without the pH gradient. Accordingly, in some embodiments, the ratio of a virus filter foulant to the purified polypeptide is at least about 20% lower, about 30% lower, about 40% lower, about 50% lower, about 60% lower, or about 70% lower than the ratio in a polypeptide purified by a step elution method, wherein the step elution method comprises binding the polypeptide to Protein A and eluting with a pH starting at or below 3.6.

In some embodiments, the purified polypeptide has virus particle or virus-like particle count less than about 15000 particles/ml. In some embodiments, the purified polypeptide has virus particle or virus-like particle count less than about 12500 particles/ml, less than about 10000 particles/ml, less than about 7500 particles/ml, less than about 5000 particles/ml, less than about 2500 particles/ml, less than about 1500 particles/ml, less than about 1000 particles/ml, less than about 750 particles/ml, less than about 500 particles/ml, less than about 250 particles/ml, less than about 100 particles/ml, or less than about 50 particles/ml. In some embodiments, the virus-like particle is a retrovirus-like particle.

As used herein, the term "virus particle" is a virion consisting of nucleic acid core surrounded by a protective coat of protein (capsid). "Virus-like particles" are non-infectious virus that resemble similar morphological, biochemical or other properties. They are defective in at least one of the components necessary for virus lifecycle. An example of virus-like particle is a retrovirus-like particle that can not replicate. Virus particle or virus-like particle can be endogenous or exogenous (adventitious). An endogenous virus particle or virus-like particle is produced by a host cell line, present in the cells and cell culture fluid, and can be considered as a host cell impurity. An exogenous or adventitious virus or virus-like particle is not derived from a host cell line.

In some embodiments, the purified polypeptide has viral clearance of a virus or a virus-like particle at least about 4 LRV (log 10 reduction of virus). In some embodiments, the purified polypeptide has viral clearance of a virus or a virus-like particle ranging from about 4 LRV to about 8 LRV. In some embodiments, the purified polypeptide has viral clearance of a virus or a virus-like particle ranging from about 4 LRV to about 7 LRV. In some embodiments, the purified polypeptide has viral clearance of a virus or a virus-like particle at about 5 LRV, about 6 LRV, about 7 LRV, or about 8 LRV. In some embodiments, the virus-like particle is a retrovirus-like particle.

As used herein, the LRV is the difference of log 10 (total virus) in the unpurified polypeptide and in the purified polypeptide.

In some embodiments, the purified polypeptide is a polypeptide monomer.

In some embodiments, a purified fraction of the polypeptide contains about or fewer than about 20 Protein A column volumes. In some embodiments, a purified fraction of the polypeptide contains about or fewer than about 15 Protein A column volumes. In some embodiments, a purified fraction of the polypeptide contains about or fewer than about 12 Protein A column volumes. In some embodiments, a purified fraction of the polypeptide contains about or fewer than about 11, about 10, about 9, about 8, about 7, about 6, about 5.5, or about 5.0 Protein A column volumes.

In some embodiments, the methods described herein remove at least two of the impurities described herein from the desired polypeptide monomer product. For example, the methods remove both an aggregate and a host cell line impurity, both an aggregate and a virus filter foulant, both an aggregate and a virus particle, both an aggregate and a virus-like particle, both an aggregate and a basic polypeptide variant, or a host cell line impurity and a virus particle, etc. In some embodiments, the methods described herein remove at least three of the impurities described herein from the

desired polypeptide monomer product. For example, the methods remove an aggregate, a host cell impurity, and a virus filter foulant, or an aggregate, a host cell impurity, and a virus particle, and a basic polypeptide variants, etc. In some embodiments, the methods described herein remove at least four of the impurities described herein from the desired polypeptide monomer product. For example, the methods remove an aggregate, a host cell impurity, a virus filter foulant, and a virus particle, or an aggregate, a host cell impurity, a virus filter foulant, and a virus-like particle. In some embodiments, the methods described herein remove at least five of the impurities described herein from the desired polypeptide monomer product. For example, the methods remove an aggregate, a host cell impurity, a virus filter foulant, a virus particle, and a virus-like particle, etc. In some embodiments, the methods described herein remove all of the impurities from the desired polypeptide product.

In some embodiments, the methods described herein do not comprise a further purification step to remove an aggregate, and the purified polypeptides have a purity of at least about 98% or about 99% monomer. Aggregate clearance normally performed on a separate ion exchange chromatography step is not required following the Protein A chromatography using the pH gradient as described above.

In some embodiments, the methods described herein do not comprise a further purification step to remove a virus filter foulant, and the purified polypeptides have a purity of at least about 98% or about 99% monomer.

In some embodiments, the purification method does not comprise a further purification step to remove a basic or an acidic polypeptide variant.

The purified polypeptide using the methods described herein may be subjected to additional purification steps either prior to, during, or following the Protein A chromatography step. Exemplary further purification steps include, but are not limited to, hydroxylapatite chromatography; dialysis; affinity chromatography using an antibody to capture the protein; hydrophobic interaction chromatography (HIC) (e.g, fractionation on a HIC); ammonium sulphate precipitation; Polyethylene glycol or polyethylene glycol derivative precipitation, anion or cation exchange chromatography; ethanol precipitation; reverse phase HPLC; chromatography on silica; chromatofocusing; SDS-PAGE, virus filtration, gel filtration, and weak partitioning chromatography.

In some embodiments, the polypeptides are further subjected to a virus filtration step. For example, a parvovirus filter can be used in the virus filtration step following the step of Protein A chromatography using a pH gradient as described herein.

In some embodiments, the polypeptides are further subjected to an ion exchange chromatography step. In some embodiments, the ion exchange chromatography step comprises a cation exchange chromatography step. In some embodiments, the ion exchange chromatography step comprises an anion exchange chromatography step. In some embodiments, the ion exchange chromatography step comprises a cation exchange chromatography step and an anion exchange chromatography step.

In some embodiments, the ion exchange chromatography step runs continuously after the Protein A chromatography step as described herein. For example, cation and anion exchange chromatography membranes can be used in place of the standard cation and/or anion exchange chromatography columns following the Protein A chromatography method described herein to achieve purified polypeptides of comparable purity and yield produced by the method of standard Protein A chromatography without the pH gradient followed by standard cation and anion exchange column chromatography steps.

In some embodiments, the methods described herein are manufacturing scale or commercial processes. As used herein, manufacturing scale or commercial processes refers to a large scale purification of protein/polypeptide, for example, at about 1 kL to about 25 kL fermentation scale protein/polypeptide product per purification process.

Polypeptides

The polypeptide or protein to be purified using the methods described herein includes, but is not limited to, antibody, immunoadhesin, or a polypeptide fused to, or conjugated with a C_H2/C_H3 region. Techniques for generating such molecules are discussed below.

Antibodies

Antibodies within the scope of the present invention include, but are not limited to: anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN®); anti-VEGF antibodies, including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors,* 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998) and V3LA; anti-MUC16 antibody; anti-CD4 antibodies such as the cM-7412 antibody (Choy et al. *Arthritis Rheum.* 39(1):52-56 (1996)) and the Ibalizumab (TNX355) antibody; anti-MET antibodies such as one-armed 5D5 anti-C-Met antibody; anti-HER2 antibodies Trastuzumab (HERCEPTIN®) (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285-4289 (1992), U.S. Pat. No. 5,725,856) and humanized 2C4 (WO01/00245, Adams et al.), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108B1, or Tositumomab (BEXXAR®); anti-IL-8 antibodies (St John et al., *Chest,* 103:932 (1993), and International Publication No. WO 95/23865); anti-prostate stem cell antigen (PSCA) antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD1 antibodies (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., *Transplant Intl.* 4:3-7 (1991), and Hourmant et al., *Transplantation* 58:377-380 (1994)); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul. 31, 1997); anti-IgE antibodies (including E25, E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No. 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No. PCT/US98/13410 filed Jun. 30, 1998, U.S. Pat. No. 5,714,338, Presta et al., *J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181); anti-Apo-2 receptor antibodies (WO 98/51793 published Nov. 19, 1998); anti-TNF-α antibodies, including cA2 (REMICADE®), CDP571 and MAK-195 (See, U.S. Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al. *J. Immunol.* 156(4):1646-1653(1996), and Dhainaut et al. *Crit. Care Med.* 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) antibodies (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human α4β7 integrin antibodies (WO 98/06248 published Feb. 19, 1998); anti-epidermal growth factor receptor (EGFR) antibodies (e.g. chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S. Pat. No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-Tac antibodies such as CHI-621 (SIMULECT® and ZENAPAX® (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al. *Nature* 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22

antibody directed against Fcγ RI as in Graziano et al. *J. Immunol.* 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al. *Cancer Res.* 55(23Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al. *Cancer Res.* 55(23): 5852s-5856s (1995); and Richman et al. *Cancer Res.* 55(23 Supp): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al. *Eur J Immunol.* 26(1): 1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al. *J. Immunol.* 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al. *Cancer Res* 55(23 Suppl):5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22 antibodies such as LL2 or LymphoCide (Juweid et al. *Cancer Res* 55(23 Suppl):5899s-5907s (1995)); anti-EpCAM antibodies such as 17-1A (PANOREX®); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO®); anti-RSV antibodies such as MEDI-493 (SYNAGIS®); anti-CMV antibodies such as PROTOVIR®; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR®; anti-CA 125 antibodies, such as OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-αvB3 antibodies, including VITAXIN®; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1).

Aside from the antibodies specifically identified above, the skilled practitioner can generate antibodies directed against an antigen of interest, e.g., using the techniques described below.

(i) Antigen Selection and Preparation

The antibody herein is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those proteins described in section (3) below. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20, CD22 and CD34; members of the ErbB receptor family such as the EGFR, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p1 50.95, VLA-4, ICAM-1, VCAM and av/β3 integrin including either a or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C, or any of the other antigens mentioned herein.

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

(ii) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to {fraction (1/10)} the original amount of antigen or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(iii) Monoclonal Antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromy-

eloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, Protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. Preferably the Protein A affinity chromatography procedure using a pH gradient described herein is used.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

Monoclonal antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552-554 (1990). Clackson et al., *Nature*, 352:624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional hybridoma techniques for isolation of monoclonal antibodies.

(iv) Humanized and Human Antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeyen et al., *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human FR for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta et al., *J. Immnol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such

germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255-258 (1993); Bruggermann et al., *Year in Immuno.*, 7:33 (1993); and Duchosal et al. *Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581-597 (1991); Vaughan et al. *Nature* Biotech 14:309 (1996)).

(v) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al. *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science*, 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. A single chain Fv fragment (scFv) can also be isolated. See WO 93/16185. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

(vi) Multispecific Antibodies

Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.*, 10:3655-3659 (1991).

According to another approach described in W096/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g., tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g., alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science*, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.*, 152:5368 (1994). Alternatively, the antibodies can be "linear antibodies" as described in Zapata et al. *Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H and V_L) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol* 147: 60 (1991).

Immunoadhesins

The simplest and most straightforward immunoadhesin design combines the binding domain(s) of the adhesin (e.g., the extracellular domain (ECD) of a receptor) with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the immunoadhesins of the present invention, nucleic acid encoding the binding domain of the adhesin will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, C_H2 and C_H3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the C_H1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics of the immunoadhesin.

In some embodiments, the adhesin sequence is fused to the N-terminus of the Fc domain of immunoglobulin G_1 (Ig G_1). It is possible to fuse the entire heavy chain constant region to the adhesin sequence. However, preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (i.e. residue **216**, taking the first residue of heavy chain constant region to be **114**), or analogous sites of other immunoglobulins is used in the fusion. In some embodiments, the adhesin amino acid sequence is fused to (a) the hinge region and or C_H2 and C_H3 or (b) the C_H1, hinge, C_H2 and C_H3 domains, of an IgG heavy chain.

For bispecific immunoadhesins, the immunoadhesins are assembled as multimers, and particularly as heterodimers or heterotetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of four basic units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each of the four units may be the same or different.

Various exemplary assembled immunoadhesins within the scope herein are schematically diagramed below:

(a) AC_L-AC_L;

(b) AC_H-(AC_H, AC_L-AC_H, AC_L-V_HC_H, or V_LC_L-AC_H);

(c) AC_L-AC_H-(AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H)

(d) AC_L-V_HC_H-(AC_H, or AC_L-V_HC_H, or V_LC_L-AC_H);

(e) V_LC_L-AC_H-(AC_L-V_HC_H, or V_LC_L-AC_H); and

(f) $(A\text{-}Y)_n$-$(V_LC_L\text{-}V_HC_H)_2$,

wherein each A represents identical or different adhesin amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_H is an immunoglobulin heavy chain constant domain;

n is an integer greater than 1;

Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed to be present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the adhesin sequences can be inserted between immunoglobulin heavy chain and light chain sequences, such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the adhesin sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the C_H2 domain, or between the C_H2 and C_H3 domains. Similar constructs have been reported by Hoogenboom, et al., *Mol. Immunol.* 28:1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an adhesin-immunoglobulin heavy chain fusion polypeptide, or directly fused to the adhesin. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the adhesin-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Pat. No. 4,816,567, issued 28 Mar. 1989.

Immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the adhesin portion in-frame to an immunoglobulin cDNA sequence. However, fusion to genomic immunoglobulin fragments can also be used (see, e.g., Aruffo et al., *Cell* 61:1303-1313 (1990); and Stamenkovic et al., *Cell* 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequences from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the "adhesin" and the immunoglobulin parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells.

Other C_H2/C_H3 Region-Containing Polypeptides

The polypeptide to be purified is one which is fused to, or conjugated with, a C_H2/C_H3 region. Such fusion polypeptides may be produced so as to increase the serum half-life of the protein and/or to facilitate purification of the protein by Protein A affinity chromatography. Examples of biologically important proteins which can be conjugated this way include renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones

or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a ner growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD1 9 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-a, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as EGFR, HER2, HER3 or HER4 receptor; and fragments of any of the above-listed polypeptides.

Expression of Polypeptides

The polypeptide to be purified using the method described herein is generally produced using recombinant techniques. The polypeptide may also be produced by peptide synthesis (or other synthetic means) or isolated from a native source.

For recombinant production of the polypeptide, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the polypeptide is readily isolated and sequenced using conventional procedures (e.g., where the polypeptide is an antibody by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence (e.g., as described in U.S. Pat. No. 5,534,615, specifically incorporated herein by reference).

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryotic cells. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, Enterobacter, Erwinia, Klebsiella, Proteus, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 Apr. 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe; Kluyveromyces* hosts such as, e.g., *K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa; Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., *Neurospora, Penicillium, Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

Suitable host cells for the expression of glycosylated polypeptide are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines include, but are not limited to, monkey kidney CV1 cells transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney cells (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci.* USA 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and human hepatoma cells (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the polypeptide used in the methods of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58:44 (1979), Barnes et al., *Anal. Biochem.* 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. No. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source.

Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the polypeptide can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the polypeptide is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells (e.g. resulting from homogenization), is removed, for example, by centrifugation or ultrafiltration. Where the polypeptide is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit.

Compositions Including Pharmaceutical Formulations Comprising Polypeptides

The invention also includes compositions, such as pharmaceutical formulations. A pharmaceutical formulation comprising the polypeptide purified by the methods of the present invention, optionally conjugated with a heterologous molecule, may be prepared by mixing the polypeptide having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 21st edition (2005)), in the form of lyophilized formulations or aqueous solutions.

The polypeptide product comprising a C_H2/C_H3 region purified by the methods described herein can have the desired degree of purity of at least about 98% monomer or at least about 99% monomer.

"Pharmaceutically acceptable" carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™, or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 21st edition (2005).

The formulation to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide variant, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(−)-3-hydroxybutyric acid.

The polypeptide purified as disclosed herein or the composition comprising the polypeptide and a pharmaceutically acceptable carrier is then used for various diagnostic, therapeutic or other uses known for such polypeptides and compositions. For example, the polypeptide may be used to treat a disorder in a mammal by administering a therapeutically effective amount of the polypeptide to the mammal.

The following examples are provided to illustrate, but not to limit, the invention.

EXAMPLES

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

Example 1

pH Gradient Elution Protein A Chromatography

Six different proteins containing the C_H2/C_H3 regions, anti-VEGF antibody #1, anti-CD20 antibody, anti-VEGF antibody #2, anti-MUC16 antibody, anti-CD4 antibody, and an armed anti-Met antibody were purified using a pH step-gradient elution method on a Protein A chromatography column. The method is outlined in Table 1.

TABLE 1

Run Phase	Specifics
Column Equilibration	Tris buffer, pH 7
Protein Load	Prefiltered HCCF, 14-37 g/L
Washes	Various buffers
pH Step Gradient Elution	pH Gradient: 35-60% B; A: Acetate, pH 5.0/B: Formate, pH 2.7
Column Regeneration	NaOH
Column Storage	Benzyl alcohol and acetate storage buffer

Experimental Procedures

A Unicorn method file was constructed using the protein load and buffer composition/pH parameters as listed in Table 1. This file was executed by a GE Healthcare AKTA (GE Healthcare) Explorer FPLC (Fast Protein Liquid Chromatography) system. FPLC was a bench-scale instrument made of plastic piping and pumps that simulated a manufacturing purification process. FPLC produced a "pH gradient" phase

by mixing two buffers in a programmed changing proportion by using a two pump (A pump and B pump) system where the flow rate was maintained and the percent of the flow each of the pumps delivered changed. Designated in the Unicorn program was one "% B" to another over a set volume (number of column volumes).

At the column equilibration phase, the column was taken out of storage solution as listed in Table 1 and prepared for loading the protein materials. At the protein loading phase, HCCF (Harvested Cell Culture Fluid) was prefiltered using a 0.2 micron pore size vacuum filter and was loaded onto the Protein A column (MABSELECT™, MABSELECT SURE™, POROS® MABCAPTURE™ A, PROSEP® Va, or PROSEP® Ultra Plus). Proteins were loaded at a density at the range of 14-37 g/L. Most runs were done at 21 g/L. At the Wash 1 phase, the Wash 1 buffer was used to push any load left in the AKTA lines onto the column. At the Wash 2 phase, impurities such as CHOP (Chinese Hamster Ovary Protein) was removed by the Wash 2 buffer. At the Wash 3 phase, the Wash 3 buffer was used to remove the Wash 2 buffer and the associated impurities from the column to prepare for the elution phase. At the pH step-gradient elution phase, a gradient formed by the precise manipulation of two pH buffers of different pHs by a two pump system was used to gradually move from one pH mix of the two buffers to another mix, set by percents. The elution parameter of "35-60% B" correlates to a pH gradient range of about 4.3-3.7. More specifically, 35% B corresponds to elution buffer pH of 4.34, buffer composition of 16.25 mM acetate, 8.75 mM formate, and buffer conductivity of 1039 uS/cm. 60% B corresponds to elution buffer pH of 3.69, buffer composition of 10 mM acetate and 15 mM formate, and buffer conductivity of 763 uS/cm. During this elution phase of most runs, fractions were taken throughout the elution and assayed using a size exclusion HPLC assay to determine monomer versus size variant (HMWS (High Molecular Weight Species), dimer, or fragment) elution behaviors. These fractions were also submitted to a CHOP assay for selected run, and all fractions were measured for protein concentration using a NanoDrop UV spectrophotometer (Thermo Fischer Scientific, Wilmington, Del.). At the regeneration phase, regeneration buffer was used to wash off any tightly bound impurities or leftover product to minimize carryover between runs. At the storage phase, the Protein A column was removed of regeneration buffer and stored in a solution that was designed to maintain column integrity over time in disuse.

Phase lengths and flow rates were measured in CVs and centimeters per hour, respectively. Flow rate was scaled by centimeters per hour (divide the flow rate in cm/hr by the bed height of the column in cm to arrive column volumes per hour, also a standard unit) due to pressure concerns.

Chromatograms were collected and analyzed by the AKTA (GE Healthcare) FPLC purification system and its associated Unicorn software package. After the column A purification run was performed, traces of the UV absorbance, pH, and conductivity (as well as other measured values or program instructions/logbooks) were accessed and examined.

a. Size Exclusion Chromatography (SEC) Assay

An analytical size exclusion chromatography (SEC) assay was run on an Agilent 1200 series HPLC (Agilent Technologies, USA, part G1329A) and used to determine the relative levels of high molecular weight species (HMWS), dimer, monomer, and fragment for collected samples. A 14.24 mL TSK G3000SWXL, 7.8 mmD×300 mmH (Tosoh Bioscience, Tokyo, Japan, part 08541) column was used. Each sample was either diluted to approximately 0.5 g/L antibody using the potassium phosphate/potassium chloride HPLC running buffer or sample injections were modified to standardize mass loaded to the assay column. All samples were prepared in Agilent HPLC 1.5 mL glass vials. Runs were 30 minutes with a 0.5 mL/min flow rate. Sample injections were adjusted so that approximately 25 ng of antibody was loaded per sample. Blanks containing the samples' respective background buffers were run with each sample set. UV 280 nm absorbance curves were analyzed either manually using ChemStation (Agilent Technologies) or automatically using CHROMELEON® (DIONEX, Sunnyvale, Calif.) software to integrate peaks separately to obtain percentage values of species for the samples. Percent values obtained from this assay can be multiplied by the concentration (mg/mL) of the fraction to get an actual concentrations or masses for each size variant species in the sample (e.g., SEC result: 4% HMWS, 3% dimer, 92% monomer, 1% fragment; sample concentration: 2 g/L; sample volume: 10 mL; 1.84 g/L monomer, 18.4 mg monomer total in sample).

b. CHOP assay

Samples from selected runs were submitted to an assay group that performed a standard and validated enzyme linked immunosorbent assay (ELISA) to quantitate the levels of CHOP. Affinity-purified goat anti-CHOP antibodies were immobilized on microtiter plate wells. Dilutions of the samples containing CHOP, standards, and controls, were incubated in the wells, followed by incubation with goat anti-CHOP antibodies conjugated to horseradish peroxidase. The horseradish peroxidase enzymatic activity was detected with o-phenylenediamine dihydrochloride. The CHOP was quantitated by reading absorbance at 492 mn in a microtiter plate reader. A computer curve-fitting program was used to generate the standard curve and automatically calculate the sample concentration. The assay range for the ELISA was typically 5 ng/ml to 320 ng/ml. Results were standardized to ppm for pool comparisons.

Results

The elution shape of a step-gradient is shown in FIG. **1**. All proteins were eluted by the end of the pH decline. A larger portion of the proteins were eluted from the column more rapidly, but enough proteins remained on the column and were eluted during the gradient. At lower pH, the separation between the desired product (elution range of around pH 4.6 to 3.7 with slight molecule to molecule variation) and undesirable aggregate (elution range around pH 3.9 to 3.5) occurred.

In all six protein molecules tested (anti-VEGF antibody #1, anti-CD20 antibody, anti-VEGF antibody #2, anti-MUC16 antibody, anti-CD4 antibody, and one-armed anti-Met antibody), high percentages (~100%) of monomer were observed in the initial gradient fractions and the tail end portion of the gradient contained much higher levels of aggregated species (>50%). These SEC results are shown in FIGS. **2**-**4**B. Since this set of tested molecules encompasses larger classes of protein molecules (e.g., chimeric antibodies, thioMabs (recombinant monoclonal antibodies having a point mutation by replacing one amino acid residue with cysteine), IgG4s, and antibody fragments produced by *E. coli*), this pH step-gradient method suggest broad applicability to all C_H2/C_H3 region-containing polypeptides/proteins (e.g., Fc region).

Example 2

CHOP Separation for Anti-CD20 Antibody, Anti-VEGF Antibody #1, and Anti-MUC16 Antibody over a Protein A Chromatography Column Using a Standard Step Elution and a pH Step-Gradient Elution

Using the method of pH step-gradient elution protein as described in Example 1, the anti-CD20 antibody levels per fraction in mg/mL (from the bench top offline UV 280 absorbance, which tracked with the online AKTA/Unicorn UV 280 readings for the step-gradient elution phase as seen on the anti-VEGF antibody #1 chromatogram in FIG. **1**A) and the CHOP levels per fraction in ppm were measured. As seen in the left panel of FIG. **5** (anti-CD20 antibody and CHOP elution), the later fractions at lower pH of the pH step-gradient elution contained very little anti-CD20 antibody compared to the amount of CHOP. Data from the control run done at the same time as the anti-CD20 antibody step-gradient pH elution is shown in the top row of the table in FIG. **5**. Control run was using the same conditions as described in Table 1, except that no pH step-gradient was used during the protein elution phase, and the protein was eluted at or below pH 3.6. The bottom row shows a small, but acceptable, decrease in the anti-CD20 antibody yield using the pH step-gradient. (note: this yield loss due to aggregate removal is expected; yield is calculated using the HCCF titer value that includes aggregates in the total amount of product loaded on the column). About 5% less aggregate and half of the CHOP levels were observed in comparison to the control pool. The results establish an unexpected benefit of increased purity using the pH step-gradient elution.

CHOP separations for both anti-VEGF antibody #1 and anti-MUC16 antibody were also performed using the pH step-gradient method described in Example 1. Similar to the pattern observed in anti-CD20 antibody CHOP graph, more CHOP was eluted at the end of the pH gradient in proportion to the elution of anti-VEGF antibody, indicating that the pH step-gradient elution separated host cell impurities for this protein molecule as well as the anti-CD20 antibody. See FIG. **6**. For CHOP separation in the anti-MUC16 antibody, this antibody had much higher CHOP levels in comparison to the elution pattern observed in anti-VEGF antibody#1. See FIG. **7**. Accordingly, a significant amount of CHOP can be fractionated from anti-MUC16 antibody by using the pH step-gradient method as described above.

Example 3

Virus Particle Clearance Using pH Gradient Elution Protein A Chromatography

Using the method of pH step-gradient elution protein A chromatography as described in Example 1, the virus particle clearance of anti-VEGF antibody #1 was measured.

Step-Gradient pH Elution Protein A Chromatography

All phases and buffers were the same as those used in Example 1. Fractions were tested for retrovirus-like particle counts using a quantitative polymerase chain reaction assay.

a. Retrovirus-Like Particle Quantitative Polymerase Chain Reaction (RVLP QPCR) Assay

The RVLP endogenous virus particle assay is a real-time quantitative PCR assay. Viral RNA was extracted from samples using Qiagen EZ1 (Qiagen, Valencia, Calif.). Sample sizes were 0.4 mL (undiluted and 1:10 diluted

HCCF, undiluted protein A pool). Extraction efficiency was confirmed by including a reference standard HCCF sample with a known CHO retrovirus particle titer. Genomic DNA was removed by DNase digestions by treating the extraction eluate with 0.2 units/mL of DNase I at an elevated temperature for 30 min. The DNase was then heat inactivated at 70° C. for 15 min. The absence of retroviral DNA was confirmed by assaying the samples without reverse transcriptase.

Real-time quantitative PCR assays to measure CHO retrovirus genomes were performed as described in De Wit et al. (Biologicals, 28(3):137-48 (2000)), but with the use of a new probe at nearby region. The reagents and procedures were also updated and improved. Primers and probe sequences were designed to amplify a fragment in the highly conserved pol region from the CHO type C retrovirus genome. Each retrovirus-like particle contains two genomic RNA molecules. Oligonucleotide probes and primers were ordered from Applied Biosystems (Foster City, Calif.) and Invitrogen (Carlsbad, Calif.). Viral clearance was expressed as log 10 reduction value, or LRV, which is the difference of log 10 (total virus) in the protein (HCCF) load and in the product pool. Total virus was obtained from virus titers (particles/ml or nU/mL) in samples and sample volume (mL) FIG. **18** shows the endogenous virus-like particle count for each fraction taken from the pH step-gradient elution. Some virus eluted at the start of the gradient in the large peak, but a larger portion of the virus eluted at the tail end of the elution. Accordingly, separation of these RVLPs from product can benefit pH step-gradient or full-gradient elution Protein A chromatography overall efficiency in terms of viral clearance. FIG. **19** shows LRV for each fraction in comparison to the HCCF load. The graph was based on a calculation using the values from FIG. **18**. A large decrease in the LRV was observed in the later aggregate rich elution fractions. LRV was high (desirable effect) in the middle eluting, higher polypeptide monomer fractions.

Example 4

Basic Polypeptide Variants Clearance Using pH Gradient Elution Protein A Chromatography

Using the method of pH step-gradient elution protein A chromatography as described in Example 1, the basic polypeptide variants (or basic variants) clearance of anti-VEGF antibody #1 was measured.

Step-Gradient pH Elution Protein A Chromatography

All phases and buffers were the same as those used in Example 1. Fractions were submitted to the ion-exchange variant assay.

a. Ion-Exchange Variant Assay

An analytical ion exchange chromatography (IEC) assay was run on an Agilent 1200 series HPLC (Agilent Technologies, USA, part G1329A) and used to determine the relative levels of main peak to acidic and basic charged variants for collected Anti-VEGF antibody #1 samples. A Dionex ProPac WCX-10, 4.6×250 mm (Dionex product no. 054993) column was used with a gradient of ACES [N-(2-Acetamido)-2-aminoethanesulfonic acid] and NaCl under conditions of elevated temperature. Sample preparation included buffer exchanging samples into IEC mobile phase prior to a 20 minute heated digestion with Carboxypeptidase (CpB). Approximately 50 μg of Anti-VEGF antibody #1 was injected into the column per sample. UV 280 nm traces were obtained and integrated using ChemStation (Agilent Technologies) software. Integration percents for each category of acidic, basic, and main peak species were analyzed for

ion-exchange variant composition trends across the gradient. FIG. **17** shows the result of the ion exchange variant assay peak integration across 20 Protein A pH step gradient elution fractions. The percent of basic variants present in the fractions increases dramatically in the tail portion of the Protein A pH step-gradient elution. This tail portion of the gradient elution is the same portion where the increased CHOP and aggregation separation were observed, as described in Examples 1 and 2.

Example 5

pH Step-Gradient Elution Using Multiple Protein A Chromatography Columns

Both the MABSELECT SURE™ and the MABSELECT™ resins were tested for the pH step-gradient elution method as described above. These two Protein A resins, although similar in name, have different affinity ligands attached. The MABSELECT™ bears the native Protein A ligand, which bind to the Fc portions of antibodies. The MABSELECT SURE™ bears a modified form of Protein A that has been chemically altered to be stable in solutions of high pH for short amounts of time. The overlayed AKTA elution profiles show that the elution traces are extremely similar for the two Protein A resins. As can be seen by the SEC integration profile for MabSelect in FIG. **8**, comparable aggregate separation was achieved using this resin. Other resins tested successfully for aggregate separation were PROSEP® Va, PROSEP® Ultra Plus, and POROS® MABCAPTURE™ A. Accordingly, various affinity resins (e.g., MABSELECT™, MABSELECT SUERE™, PROSEP® Va, PROSEP® Ultra Plus, and POROS® MABCAPTURE™) can be used in the pH step-gradient elution method to fractionate impurities.

Example 6

Design of Experiments (DOE) Using Various Parameters for the pH Gradient Elution Method

Various parameters that are important in most chromatography processes were explored for anti-VEGF antibody #1 using a 35-run statistically designed study within the ranges shown in Table 2. The "elution start % B" parameter affects the starting pH of the elution phase (which plays a major role in determining the shape of the elution curve. The higher the starting % B, the lower the starting elution pH, the more protein eluted from the column in the first fractions), as well as the slope of the overall gradient. Parameters were varied simultaneously in a fractional factorial study designed to elucidate the main effects as well as the interactions. All runs were fractionated during the elution, and aggregate and concentration were assayed for all fractions. An interpolative calculation was used to determine the mock pool monomer levels for pools that would result in exactly 85% yield (the lower limit of the step yield target) and these values were used to compare the effectiveness of each set of run parameters in separating monomer from aggregate efficiently.

TABLE 2

DOE Parameter Ranges			
Parameter	Low	Target	High
Load Density	19	28	37
Bed Height	14 cm	22 cm	30 cm
Elution Flow Rate	140 cm/hr	210 cm/hr	350 cm/hr
Elution Length	15 CV	20 CV	25 CV
Elution Start % B (pH)*	25 (4.6)	35 (4.3)	45 (4.2)

*All elutions ended at 60% B (pH 3.7)

Experimental Procedures

All parameter changes aside from the column bed height (which requires packing of multiple columns) were examined using the Unicorn software. CV fractions were taken throughout all of the elutions, assayed using the HPLC SEC (as described above), and measured for the protein concentration (UV 280 absorbance as measured on a NanoDrop UV spectrophotometer). In order to best standardize the results for comparison, data from various sets of fractions were compiled and a calculation was used to interpolate the overall yield and SEC profile of a pool from each of the run.

The JMP® (SAS, Cary, N.C.) software package was employed to generate a fractional factorial parameter exploration run plan. One of the runs was selected as the "exemplary manufacturing run" from the set because of its high yield, high monomer level, and low pool size (FIG. **11**).

Results

Pareto plot of DOE results show that the "Start % B" is the most influential parameter in determining aggregate separation, followed by load density (lower is better) and residence time (residence time was calculated by dividing the controlled parameters of bed height (cm/CV) by flow rate (cm/hr) for hr/CV. Accordingly, the lower the start % B, the higher the elution start pH, and the more efficient the aggregate separation; the lower the load density, the more efficient the aggregate separation; and the higher the residence time, the more efficient the aggregate separation. See FIG. **9**. Further, interaction profiles from this study shows that there was an interaction between the parameters of load density and start % B as well as an interaction between the residence time and load density. At increased load densities, the start % B had a greater effect on the monomer levels of 85% yield mock pools than if the run was done at lower load densities. Further, at increased load density, the residence time played a much larger role in the ability of the gradient to fractionate aggregates more efficiently. A lower rate must be used during the elution to achieve high product throughput with the pH step-gradient method. See FIG. **10**. FIG. **11** shows that the pool from this run was under 10 CVs (e.g., 5.4 CV or 6 CV) while delivering less than 1% aggregate with greater than 85% yield.

In addition to the main effects (Pareto plot in FIG. **9**), interactions (FIG. **10**), and exemplary manufacturing run (FIG. **11**), it was observed that overall elution length had no effect on aggregate separation, but could be manipulated to decrease the pool size to result in a pool that still has high purity and high yield, but in a lesser volume (which is preferable for manufacturing scale). Also, the use of the pH step-gradient resulted in lower pool volumes with purity nearly comparable to that given by the full gradient (see Example 8). The pH step-gradient was also found to be robust in several process changes, including smaller changes in load density and flow rate, as well as being completely unaffected by bed height.

Example 7

Protein Purification Using Ion Exchange Membrane Chromatography Following the pH Step-Gradient Protein A Chromatography

To determine whether downstream column chromatographies could be eliminated from the purification process or substituted with membranes, bench scale cycling of the Protein A pH step-gradient on anti-VEGF antibody #1 (with a pool of less than 1% aggregate and a high yield) was loaded onto downstream charged membranes. MUSTANG® S (Pall corporation) and MUSTANG® Q (Pall corporation) membranes represent a cation exchange membrane and an anion exchange membrane, respectively. Success was measured by the ability of the membranes to achieve the same overall purities and yields as compared to the typical downstream column process.

Experimental Procedures

Parameters used for determining optimal load conditioning for CHOP and aggregate clearance over S and/or Q membranes were taken from prior studies on optimal load conditioning for CHOP clearance over S and/or Q membrane using a Protein A standard step pool in Tables 3A and 3B. In these prior studies, promising results were shown when a Protein A standard step pool was used (control group, using no pH gradient and eluting protein at or below pH 3.6); however, this process had slightly higher CHOP levels than desired and did not clear any aggregates from the process. In these prior studies, membranes had been loaded to 5 kg/L membrane and optimal loading conditions had been found for impurity removal. These same conditions were used for the Protein A step-gradient elution pool to compare the performance of the different Protein A pools on these membranes with the primary impurities targeted as CHOP and aggregate.

TABLE 3A

Prior parameters for determination of optimal load conditioning for CHOP clearance over S or Q membranes

Filter	MUSTANG ® S or Q ACRODISC ®
Filter Size	0.18 mL
Load	anti-VEGF antibody #1 Protein A Step Pool
Load Density	5 kg/L membrane
Flow Rate	4 mL/min (1333 mV/hr, 112 cm/hr)
Assay	CHOP ELISA

TABLE 3B

Prior parameters for determining whether orthogonal impurity clearance can be achieved by running S and Q membranes in series.

Best MUSTANG ® S pool (Protein A Step Pool as load)	load at pH: 5-6, 3 mS/cm: 140 ppm CHOP
Best MUSTANG ® Q pool (Protein A Step Pool as load)	load at pH: 8-9, <4 mS/cm; 30 ppm CHOP
Best MUSTANG ® Q pool (Best MUSTANG S Pool as load)	load at pH: 8-9, 2.5 mS/cm; 15-20 ppm CHOP

In the initial studies, Protein A standard step elution pools were conditioned to specific pHs and conductivities and passed through laboratory scale cation (MUSTANG® S) and anion (MUSTANG® Q) exchange membrane units that were connected to the AKTA™ FPLC purification system. Flow rates were maintained and pressure traces were examined for evidence of fouling/permeability decay. Fractions were taken at various load densities and assayed for CHOP (ELISA) and antibody concentrations (UV 280 absorbance on the NanoDrop UV spectrophotometer). The results of these assays were compared at various load densities to find the optimal loading conditions for end CHOP clearance. In the later studies, the Protein A pH step-gradient elution pool was conditioned to the optima found in the initial studies. From this, the performance of the step-gradient elution pool was compared to that of the standard Protein A step elution in ability of the downstream membranes to clear CHOP and other impurities while maintaining high yield and low aggregate levels. Two sizes of cation exchange membrane and three sizes of anion exchange membrane were also used to test reproducibility and scalability of results.

Results

When the Protein A step pool was used as the load for a cation to anion exchange membrane purification series, the lowest CHOP levels obtained were 15-20 ppm for membranes loaded to 5 kg/L membrane. Since the membranes did not clear aggregate, levels of this impurity were unacceptably high in the end pools. In contrast, when the Protein A pH step-gradient elution pool was loaded onto these same membranes, CHOP levels were 0-15 ppm and aggregate levels were still low in the end pools, showing a benefit to the overall process.

Further, end pools of each of the three combinations of ion exchange membranes tested resulted in high yield and high purity end pools. In all cases, the aggregate levels remained low through pH adjustment and membrane processing, and CHOP levels were also lower than what was seen in the prior control studies, showing an unexpected CHOP reduction benefit to using a lower aggregate protein A pool as a feed. See FIG. **12**. Accordingly, the use of ion exchange chromatography membranes in place of the usual ion exchange chromatography columns eliminates the need for many typical column chromatography buffers and other time/manufacturing space consuming inconveniences. More importantly, since these charged membranes were used in overload mode (i.e., the load is passed through the membrane to a high loading density with no washing or elution steps needed to generate a highly purified pool), the membranes allow for a continuous purification process downstream of the pH gradient Protein A chromatography step.

The SEC integration profiles between the pilot scale (4.1 L) and the 28 mL bench scale are also shown to be extremely similar, indicating that Protein A pH step-gradient can be scaled successfully and that any small scale results can be considered indicative of larger scale performance. See FIG. **13**. These results indicate an unexpected benefit of reproducibility and scalability of pH step-gradient elution Protein A chromatography.

Example 8

VIRESOLVE® Pro Parvovirus Filter Runs

A. VIRESOLVE® Pro Permeability Decay Comparison

The Protein A pH step-gradient was compared to the Protein A standard step (control group, using no pH gradient and eluting protein at or below pH 3.6) in terms of facilitating a greater mass throughput over a parvovirus filter (VIRESOLVE® Pro, Millipore, Inc.). A common HCCF feed was used to run both the Protein A pH step-gradient and Protein A standard step control over the QSFF (Q Sepharose Fast Flow (anion exchange column; GE Healthcare) in a standard flow through mode before running over the VIRE-

SOLVE® Pro parvovirus filter. Several VIRESOLVE® Pro run conditions were tested: 1) Protein A standard step elution pool with the SHC sterile prefilter in-line, 2) Protein A standard step elution with a cation exchange (CEX) membrane adsorber as a prefilter in-line, 3) the Protein A pH step-gradient pool with the SHC prefilter (an uncharged 0.2 micron sterile-grade filter), and 4) the Protein A pH step-gradient pool with a CEX membrane adsorber as a prefilter in-line. Some pools were run with repetition. The VIRESOLVE® Pro was run on a filtration setup that utilizes a set of peristaltic pumps, balances, and pressure sensors to report data into a spreadsheet

The pH step-gradient pool with the SHC sterile filter in line performed similarly to the step pool with the cation exchange membrane, both showing around a six-fold increase in possible mass throughput over the VIRESOLVE® Pro. See FIG. **14**. Accordingly, this result indicates the unexpected benefit of the protein A pH step-gradient in removing VIRESOLVE® Pro filter foulants without the aid of a CEX membrane prefilter. Additionally, the VIRESOLVE® Pro performance benefited by the combination of the CEX membrane adsorber with the Protein A pH step-gradient pool, leading to an unexpected approximate 18-fold improvement in potential mass throughput as compared to the Protein A standard step elution pool run with the SHC on the VIRESOLVE® Pro.

B. VIRESOLVE® Pro CHOP and SEC Experiments

Samples were taken at different points during the VIRESOLVE® Pro run sequences and assayed for CHOP and SEC. The Protein A standard step sequence with the SHC in line had fairly low CHOP levels, but still contained aggregates. Aggregate levels remained low in the Protein A pH step-gradient experiments through different process steps and resulted in an end pool with less than 1% aggregate. This result was a significant improvement over the pool delivered by the standard step pool. Further, the CHOP levels for the pH step-gradient SHC-VIRESOLVE® Pro pools were comparable to the levels in the standard step run with the cation exchange membrane. The levels were slightly lower than those from the standard step with the SHC. See Tables 4A and 4B. These results show that not only did the pH step-gradient method produced higher mass throughput in comparison to the standard step pool, it also produced comparable or better purity.

TABLE 4A

	Protein A STEP Elution					
	Experiment 1			Experiment 2		
HCCF Load CHOP: 1,000,000 ppm	CHOP ppm	Monomer	Aggregate	CHOP ppm	Monomer	Aggregate
Protein A Pool				1090	95.39	4.61
QSFF Load	1000	94.01	5.99	1080	94.04	5.96
QSFF Pool	9	95.53	4.47	14	95.28	4.72
VPro Load	9	95.48	4.52			
VPro Pool (SHC)	8	95.52	4.48	12	95.29	4.71
VPro Pool (CEX Membrane)	3	95.52	4.48	6	95.43	4.57

TABLE 4B

	Protein A pH STEP-GRADIENT Elution					
	Experiment 1			Experiment 2		
HCCF Load CHOP: 1,000,000 ppm	CHOP ppm	Monomer	Aggregate	CHOP ppm	Monomer	Aggregate
Protein A Pool	800	99.28	0.72	920	99.42	0.58
QSFF Load	850	98.70	1.30			
VPro Load	5	99.29	0.71			
VPro Pool (SHC)	7	99.27	0.73	6	99.32	0.68
VPro Pool (CEX Membrane)				<4	99.33	0.67

Example 9

Protein A Full pH Gradient Elution on Protein A

Protein A full pH gradient elution was also tested. All phases and buffers were the same as those used in the pH step-gradient method as described in Example 1, except that 35-60% B as used in the pH step-gradient was decreased to 25-60% at the start of the gradient, which resulted in a higher pH start gradient (starting at about pH 4.6 and ending at about pH 3.7). 25% B correspond to elution buffer pH of 4.58, buffer composition of 18.75 mM acetate and 6.25 mM formate, and buffer conductivity of 1141 uS/cm. 60% B corresponds to elution buffer pH of 3.69, buffer composition of 10 mM acetate and 15 mM formate, and buffer conductivity of 763 uS/cm. See Table 5.

TABLE 5

Run Phase	Specifics
Column Equilibration	Tris buffer, pH 7
Protein Load	Prefiltered HCCF, 14-37 g/L
Washes	Various buffers
pH Full Gradient Elution	pH Gradient: 25-60% B; A: Acetate, pH 5.0/ B: Formate, pH 2.7
Column Regeneration	NaOH
Column Storage	Benzyl alcohol and acetate storage buffer

The elution shape of a full gradient is shown in FIG. **15**. All products were eluted by the end of the pH decline. The

chromatogram for the full pH gradient is the same in all phases as the pH step-gradient, except that the antibody elution begins at a higher pH. The lack of the initial tall UV 280 spike at the start of the gradient resulted in lower concentration fractions at the beginning of the gradient, with more protein distributed through the rest of the elution phase. This allowed for greater separation of species that elute preferentially at higher pHs than the monomer. Thus, this technique can be equally effective at clearing impurities that separate from the desired product at lower pHs (i.e. the aggregates and CHOPs separated using the step-gradient), with the only drawback as end pool volume as compared to the pH step-gradient (lower fraction concentrations at the start of the gradient translate into a need to pool more fractions together to achieve a mock pool of a desired yield). The SEC traces and integrations for this full gradient also demonstrate separation of aggregate from monomer at the lower pHs, suggesting that benefits and applicability of the pH step-gradient can be extended to a full gradient as well.

Example 10

Aggregate Formation on Protein A Study

To ensure that an aggregate was neither being formed during the low pH or the tail end of the pH step-gradient elution and that the Protein A pH step-gradient technique indeed separated a monomer from an aggregate, rather than causing the formation of an aggregate, purified materials were used to show that aggregate levels of the feed were not increased by processing over Protein A, with or without HCCF components. See Table 6.

TABLE 6

Protein A Load Material	anti-VEGF antibody #1 HCCF (~pH 7.3)	Protein A pH step-gradient pool, conditioned to pH 7.3	50/50 Mix: HCCF flow through + Protein A pH step-gradient pool
Load Monomer	Unknown*	98.88%	Unknown*
Acetic Acid Step Elution Monomer	95.03%	99.06%	99.16%

*SEC monomer assay not used on HCCF

What is claimed is:

1. A method for purifying a polypeptide comprising a C_H2/C_H3 region, comprising the steps of:

(a) binding the polypeptide to Protein A; and

(b) eluting the polypeptide with a pH gradient starting at or below 5.0 using an elution buffer, wherein the elution buffer contains a high pH buffer and a low pH buffer and wherein the pH gradient is formed by adjusting a percentage of each pH buffer in the elution buffer,

wherein the high pH buffer is at about 5.0 and wherein the low pH buffer is at about 2.7, wherein the pH gradient ends at about 3.7, and

wherein an aggregate, a host cell impurity, a virus filter foulant, a virus particle and a virus-like particle are removed from the desired polypeptide.

2. The method of claim 1, wherein the percentage of low pH buffer starts at about 35%.

3. The method of claim 2, wherein the elution buffer containing the low pH buffer at about 35% comprises about 16.25 mM acetate and about 8.75 mM formate.

4. The method of claim 1, wherein the percentage of low pH buffer starts at about 25%.

5. The method of claim 4, wherein the elution buffer containing the low pH buffer at about 25% comprises about 18.75 mM acetate and 6.25 mM formate.

6. The method of claim 1, wherein the percentage of low pH buffer starts at about 40%.

7. The method of claim 6, wherein the elution buffer containing the low pH buffer at about 40% comprises about 15 mM acetate and 10 mM formate.

8. The method of claim 1, wherein the polypeptide is loaded with a loading density starting at about 14 g/L.

9. The method of claim 1, wherein the Protein A is a Protein A column chromatography resin or a Protein A chromatography sorbent.

10. The method of claim 9, wherein the Protein A chromatography sorbent is a membrane or a monolith.

11. The method of claim 9, wherein the Protein A is a Protein A column chromatography resin and wherein the polypeptide has an elution flow rate ranging from about 5 column volume/hour to about 25 column volume/hour.

12. The method of claim 9, wherein the Protein A is a Protein A column chromatography resin and wherein a purified fraction of the polypeptide contains about or fewer than about 12 Protein A column volumes.

13. The method of claim 1, wherein the pH gradient starts at about pH 4.2.

14. The method of claim 1, wherein the pH gradient starts at about pH 4.3.

15. The method of claim 14, wherein the polypeptide is an anti-VEGF antibody, an anti-CD20 antibody, an anti-MUC16 antibody, an anti-MET antibody or an anti-CD4 antibody.

16. The method of claim 15, wherein the antibody is an anti-VEGF antibody.

17. The method of claim 16, wherein the anti-VEGF antibody is bevacizumab.

18. The method of claim 15, wherein the antibody is an anti-CD20 antibody.

19. The method of claim 18, wherein the anti-CD20 antibody is rituximab.

20. The method of claim 1, wherein the pH gradient starts at about pH 4.6.

21. The method of claim 1, wherein the host cell impurity is Chinese Hamster Ovary Protein (CHOP).

22. The method of claim 1, wherein a basic polypeptide variant is separated from the polypeptide.

23. The method of claim 22, wherein the polypeptide is an anti-VEGF antibody, an anti-CD20 antibody, an anti-MUC16 antibody, an anti-MET antibody or an anti-CD4 antibody.

24. The method of claim 23, wherein the antibody is an anti-VEGF antibody.

25. The method of claim 24, wherein the anti-VEGF antibody is bevacizumab.

26. The method of claim 23, wherein the antibody is an anti-CD20 antibody.

27. The method of claim 26, wherein the anti-CD20 antibody is rituximab.

28. The method of claim 1, wherein the C_H2/C_H3 region comprises a Fc region of an immunoglobulin.

29. The method of claim 1, wherein the polypeptide is an antibody.

30. The method of claim 29, wherein the antibody is a monoclonal antibody, a polyclonal antibody, a multi-specific antibody, or an antibody fragment.

31. The method of claim **1**, wherein the polypeptide is an immunoadhesion.

32. The method of claim **1**, wherein the purified polypeptide has a purity of at least about 98% monomer.

33. The method of claim **32**, wherein the polypeptide is an anti-VEGF antibody, an anti-CD20 antibody, an anti-MUC16 antibody, an anti-MET antibody or an anti-CD4 antibody.

34. The method of claim **33**, wherein the antibody is an anti-VEGF antibody.

35. The method of claim **34**, wherein the anti-VEGF antibody is bevacizumab.

36. The method of claim **33**, wherein the antibody is an anti-CD20 antibody.

37. The method of claim **36**, wherein the anti-CD20 antibody is rituximab.

38. The method of claim **1**, wherein the purified polypeptide has a purity of at least about 99% monomer.

39. The method of claim **38**, wherein the polypeptide is an anti-VEGF antibody, an anti-CD20 antibody, an anti-MUC16 antibody, an anti-MET antibody or an anti-CD4 antibody.

40. The method of claim **39**, wherein the antibody is an anti-VEGF antibody.

41. The method of claim **40**, wherein the anti-VEGF antibody is bevacizumab.

42. The method of claim **39**, wherein the antibody is an anti-CD20 antibody.

43. The method of claim **42** wherein the anti-CD20 antibody is rituximab.

44. The method of claim **1**, wherein a ratio of a host cell impurity to the purified polypeptide is at least about 20% lower than the ratio in a polypeptide purified by a step elution method, wherein the step elution method comprises binding the polypeptide to Protein A and eluting with a pH starting at or below 3.6.

45. The method of claim **44**, wherein the polypeptide is an anti-VEGF antibody, an anti-CD20 antibody, an anti-MUC16 antibody, an anti-MET antibody or an anti-CD4 antibody.

46. The method of claim **45**, wherein the antibody is an anti-VEGF antibody.

47. The method of claim **46**, wherein the anti-VEGF antibody is bevacizumab.

48. The method of claim **45**, wherein the antibody is an anti-CD20 antibody.

49. The method of claim **48**, wherein the anti-CD20 antibody is rituximab.

50. The method of claim **1**, wherein a ratio of a host cell impurity to the purified polypeptide is at least about 60% lower than the ratio in a polypeptide purified by a step elution method, wherein the step elution method comprises binding the polypeptide to Protein A and eluting with a pH starting at about 3.6.

51. The method of claim **50**, wherein the polypeptide is an anti-VEGF antibody, an anti-CD20 antibody, an anti-MUC16 antibody, an anti-MET antibody or an anti-CD4 antibody.

52. The method of claim **51**, wherein the antibody is an anti-VEGF antibody.

53. The method of claim **52**, wherein the anti-VEGF antibody is bevacizumab.

54. The method of claim **51**, wherein the antibody is an anti-CD20 antibody.

55. The method of claim **54**, wherein the anti-CD20 antibody is rituximab.

56. The method of claim **44** or **50**, wherein the purified polypeptide is a polypeptide monomer.

57. The method of claim **1**, wherein the Protein A is a modified or a non-modified Protein A ligand.

58. The method of claim **1**, wherein the purification is a manufacturing scale process.

59. The method of claim **1**, further comprising subjecting the polypeptide to a virus filtration step.

60. The method of claim **1**, further comprising subjecting the polypeptide to an ion exchange chromatography step.

61. The method of claim **60**, wherein the ion exchange chromatography step runs continuously after step (b).

62. The method of claim **1**, wherein the method does not comprise a further purification step to remove an aggregate.

63. The method of claim **1**, wherein the method does not comprise a

further purification step to remove a virus filter foulant.

64. The method of claim **63**, wherein the polypeptide is an anti-VEGF antibody, an anti-CD20 antibody, an anti-MUC16 antibody, an anti-MET antibody or an anti-CD4 antibody.

65. The method of claim **64**, wherein the antibody is an anti-VEGF antibody.

66. The method of claim **65**, wherein the anti-VEGF antibody is bevacizumab.

67. The method of claim **64**, wherein the antibody is an anti-CD20 antibody.

68. The method of claim **67**, wherein the anti-CD20 antibody is rituximab.

69. The method of claim **1**, wherein the polypeptide is an anti-VEGF antibody, an anti-CD20 antibody, an anti-MUC16 antibody, an anti-MET antibody or an anti-CD4 antibody.

70. The method of claim **69**, wherein the antibody is an anti-VEGF antibody.

71. The method of claim **70**, wherein the anti-VEGF antibody is bevacizumab.

72. The method of claim **69**, wherein the antibody is an anti-CD20 antibody.

73. The method of claim **72**, wherein the anti-CD20 antibody is rituximab.

* * * * *

EXHIBIT W

US006586206B1

(12) **United States Patent**
Dixit et al.

(10) **Patent No.: US 6,586,206 B1**
(45) **Date of Patent: Jul. 1, 2003**

(54) **METHODS FOR MAKING RECOMBINANT PROTEINS USING APOPTOSIS INHIBITORS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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(52) **U.S. Cl.** **435/69.1**; 435/69.2; 435/325; 435/358; 435/465

(58) **Field of Search** 435/69.1, 69.2, 435/325, 358, 465; 935/33, 44

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,072,047 A 6/2000 Rauch et al. 536/23.5

FOREIGN PATENT DOCUMENTS

WO	WO 93/16192	8/1993
WO	WO 97/44443	11/1997
WO	WO 97/44444	11/1997
WO	WO 98/10778	3/1998
WO	WO 98/35986	8/1998
WO	WO 01/18185	3/2001

OTHER PUBLICATIONS

Al–Rubeal and Singh, "Apoptosis in cell culture" *Curr. Opin. Biotech.* 9:152–156 (1998).
Ashkenazi and Dixit, "Death receptors: signaling and modulation" *Science* 281(5381):1305–1308 (1998).
Baldwin, A., "The NF–κB and IκB Proteins: New Discoveries and Insights" *Ann. Rev. Immunol.* 14:649–683 (1996).
Barr and Tomei, "Apoptosis and Its Role in Human Disease" *Bio/Technology* 12:487–493 (1994).
Beidler et al., "The Baculovirus p35 Protein Inhibits Fas– and Tumor Necrosis Factor–induced Apoptosis" *Journal of Biological Chemistry* 270:16526–16528 (1995).
Boldin et al., "Involvement of Mach, a Novel MORT1/FADD–Interacting Protease, in Fas/APO–1– and TNF Receptor–Induced Cell Death" *Cell* 85:803–815 (1996).
Boldin et al., "Self–Association of the "Death Domains" of the p55 Tumor Necrosis Factor (TNF) Receptor and Fas/APO1 Prompts Signaling for TNF and Fas/APO1 Effects" *Journal of Biological Chemistry* 270:387–391 (1995).
Chinnaiyan and Dixit, "The Cell–Death Machine" *Current Biology* 6:555–562 (1996).
Chinnaiyan et al., "FADD, a novel death domain–containing protein, interacts with the death domain of Fas and initiates apoptosis" *Cell* 81:505–512 (1995).
Chinnaiyan et al., "FADD/MORT1 Is a Common Mediator of CD95 (Fas/APO–1) and Tumor Necrosis Factor Receptor–induced Apoptosis" *Journal of Biological Chemistry* 271:4961–4965 (1996).
Chinnaiyan et al., "Interaction of CED–4 with CED–3 and CED–9: A Molecular Framework for Cell Death" *Science* 275:1122–1126 (1997).
Clem et al., "Prevention of Apoptosis by Baculovirus Gene During Infection of Insect Cells" *Science* 254:1388–1390 (1991).
Cleveland and Ihle, "Contenders in FasL/TNF Death Signaling" *Cell* 81:479–482 (1995).
Dickson, A. J., "Apoptosis regulation and its applications to biotechnology" *TIBTECH* 16:339–342 (1998).
Duan et al., "ICE–LAP6, a Novel Member of the ICE–Ced–3 Gene Family, Is Activated by the Cytotoxic T Cell Protease Granzyme B" *Journal of Biological Chemistry* 271(28):16720–16724 (1996).
Enari et al., "Involvement of an ICE–like protease in Fas–mediated Apoptosis" *Nature* 375:78–81 (1995).
Franek and Chladkova–Sramkova, "Apoptosis and nutrition: Involvement of amino acid transport system in repression of hybridoma cell death" *Cytotechnology* 18:113–117 (1995).
Fraser and Evan, "A License to Kill" *Cell* 85:781–784 (1996).
Goswami et al., "Apoptosis in Batch Cultures of Chinese Hamster Ovary Cells" *Biotechnol. Bioeng.* 62:632–640 (1999).
Hsu et al., "TRADD–TRAF2 and TRADD–FADD interactions define two distinct TNF receptor 1 signal transduction pathways" *Cell* 84:299–308 (1996).
Itoh et al., "Overexpression of bcl–2, Apoptosis Suppressing Gene: Prolonged Viable Culture Period of Hybridoma and Enhanced Antibody Production" *Biotechnol. Bioeng.* 48:118–122 (1995).
Itoh et al., "The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis" *Cell* 66:233–243 (1991).
Mastrangelo and Betenbaugh, "Overcoming apoptosis: new methods for improving protein–expression systems" *TIBTECH* 16:88–95 (1998).
Mercille and Massie, "Induction of Apoptosis in Nutrient-Deprived Cultures of Hybridoma and Myeloma Cells" *Biotechnol. Bioeng.* 44:1140–1154 (1994).

(List continued on next page.)

Primary Examiner—Gabrielle Bugaisky
(74) *Attorney, Agent, or Firm*—Diane L. Marschang

(57) **ABSTRACT**

The invention provided improved methods of making and producing recombinant proteins in in vitro cultures of host cells using apoptosis inhibitors. The use of one or more apoptosis inhibitors in the methods can reduce apoptosis in the cell cultures and markedly improve yield of the desired recombinant proteins.

21 Claims, 15 Drawing Sheets

OTHER PUBLICATIONS

Murray et al., "NSO Myeloma Cell Death: Influence of bcl–2 Overexpression" *Biotech. Bioeng.* 51:(3):298–304 (1996).

Muzio et al., "FLICE, A Novel FADD–Homologous ICE/CED–3–like Protease, Is Recruited to teh CD95 (Fas/APO–1) Death–Inducing Signaling Complex" *Cell* 85:817–827 (1996).

Nagata, S., "Apoptosis by Death Factor" Cell 88:355–365 (1997).

Pan et al., "Caspase–9, Bcl–X_L, and Apaf–1 Form a Ternary Complex" *Journal of Biological Chemistry* 273(10):5841–5845 (1998).

Presta et al., "Humanization of an Antibody Directed Against IgE" *J. Immunol.* 151(5):2623–2632 (Sep. 1, 1993).

Ray et al., "Viral Inhibition of Inflammation: Cowpox Virus Encodes an Inhibitor of the Interleukin–1β Converting Enzyme" *Cell* 69:597–604 (May 15, 1992).

Shak et al., "Recombinant Human DNase I Reduces the Viscosity of Cystic Fibrosis Sputum" *Proc. Natl. Acad. Sci. USA* 87(23):9188–9192 (Dec. 1990).

Simpson et al., "Prevention of Hybridoma Cell Death by bcl–2 During Suboptimal Culture Conditions" *Biotechnol. Bioeng.* 54:1–16 (1997).

Singh et al., "Cell Death in Bioreactors: A Role for Apoptosis" *Biotechnol. Bioeng.* 44:720–726 (1994).

Singh et al., "Enhancement of Survivability of Mammalian Cells by Overexpression of the Apoptosis–Suppressor Gene bcl–2" *Biotechnol. Bioeng.* 52:166–175 (1996).

Steller, H., "Mechanisms and Genes of Cellular Suicide" *Science* 267:1445–1449 (1995).

Suzuki et al., "Establishing apoptosis resistant cell lines for improving protein productivity of cell culture" *Cytotechnology* 23:55–59 (1997).

Tartaglia et al., "A novel domain within the 55 kd TNF receptor signals cell death" *Cell* 74(5):845–853 (1993).

Tewari and Dixit, "Fas– and Tumor Necrosis Factor–induced Apoptosis Is Inhibited by the Poxvirus crmA Gene Product" *Journal of Biological Chemistry* 270:3255–3260 (1995).

Tewari and Dixit, "Recent Advances in Tumor Necrosis Factor and CD40 Signaling" *Curr. Op. Genet. Develop.* 6:39–44 (1996).

Tewari et al., "Yama/CPP32β, a Mammalian Homolog of CED–3, Is a CrmA–Inhibitable Protease That Cleaves the Death Substrate Poly(ADP–Ribose) Polymerase" *Cell* 81:801–809 (1995).

van de Goor et al., "Inhibition of Apoptosis in Biofactors by "Anti–Apoptotic Genes"" *Cold Spring Harbor Meeting on Programmed Cell Death* (abstract only) pps. 228 (1999).

Verma et al., "Rel/NF–κB/IκB Family: Intimate Tales of Association and Dissociation" *Genes Develop.* 9:2723–2735 (1995).

Zanghi et al., "Serum Protects Protein–Free Competent Chinese Hamster Ovary Cells Against Apoptosis Induced by Nutrient Deprivation in Batch Culture" *Biotech. Bioeng.* 64:108–119 (1999).

Zou et al., "Apaf–1, a Human Protein Homologous to *C. elegans* CED–4, Participates in Cytochrome c–Dependent Activation of Caspase–3" *Cell* 90:405–413 (1997).

Database WPI Section Ch. Week 199735, Derwent Publications Ltd., London, GB; Class B04, AN 1997–380167 (Jun. 24, 1997).

Fujita et al., "Overexpression of bcl–2 Improved Survival of COS–1 Cells and Enhanced Transient Protein Production" *Journal of Fermentation and Bioengineering* 82(6):589–591 (1996).

Fujita et al., "Reinforcing apoptosis–resistance of COS and meyloma cells by transfecting with bcl–2 gene" *Cytotechnology* 25:25–33 (Feb. 1997).

Hedge et al., "Blk, a BH3–containing Mouse Protein That Interacts with Bcl–2 and Bcl–xL, Is a Potent Death Agonist" *The Journal of Biological Chemistry* 273(14):7783–7786 (Apr. 3, 1998).

Li et al., "Cytochrome c and dATP–Dependent Formation of Apaf–1/Caspase–9 Complex Initiates as Apoptotic Protease Cascade" *Cell* 91:479–489 (Nov. 14, 1997).

Mercille and Massie, "Apoptosis–Resistant E1B–19K–Expressing NS/0 Myeloma Cells Exhibit Increased Viability and Chimeric Antibody Productivity under Perfusion Culture Conditions" *Biotechnology and Bioengineering* 63(5):529–543 (Jun. 5, 1999).

Mercille et al., "Dose–Dependent Reduction of Apoptosis in Nutrient–Limited Cultures of NS/O Myeloma Cells Transfected with the E1B–19K Adenoviral Gene" *Biotechnology and Bioengineering* 63(5):516–528 (A 1999).

Perkins et al., "Overexpression of Apaf–1 Promotes of Untreated and Paclitaxel– or Etoposide–treated HL–60 Cells" *Cancer Research* 58:4561–4566 (Oct. 15, 1998).

Seol and Billiar, "A Caspase–9 Variant Missing the Catalytic Site Is an Endogenous Inhibitor of Apoptosis" *The Journal of Biological Chemistry* 274(4):2072–2076 (Jan. 22, 1999).

Terada et al., "Improvement of Mammalian Cell Survival by Apoptosis–Inhibiting Genes and Caspase Inhibitors for Effective Use of Mammalian Cells" *Seibutsu–Kogaku Kaishi* 77(1):2–11 (Jan. 25, 1999).

tsang, "Mammalian Expression Vector with Two Multiple Cloning Sites for Expression of Two Foreign Genes" *Biotechniques* 22(1):68 (1997).

Zhou et al., "Fed–Batch Culture of Recombinant NSO Myeloma Cells with High Monoclonal Antibody Production" *Biotechnology of Bioengineering* 55(5):783–792 (Sep. 5, 1997).

pFLAG–CMV–2 Expression Vector, SIGMA Product Information.

Soo et al., "Overexpression of bcl–2 inhibits the apoptosis in rCHO" Abstracts of Papers American Chemical Society. 219th Meeting of The American Chemical Society, San Francisco, Mar. 26–30, 2000 219(1–2) (Abstract BIOT 245).

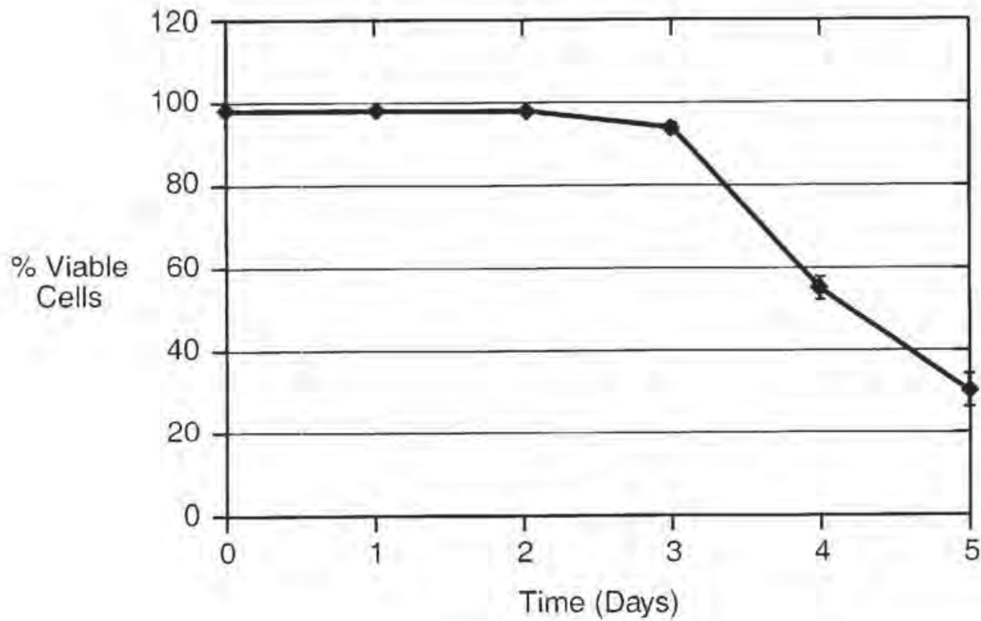

FIG._1A

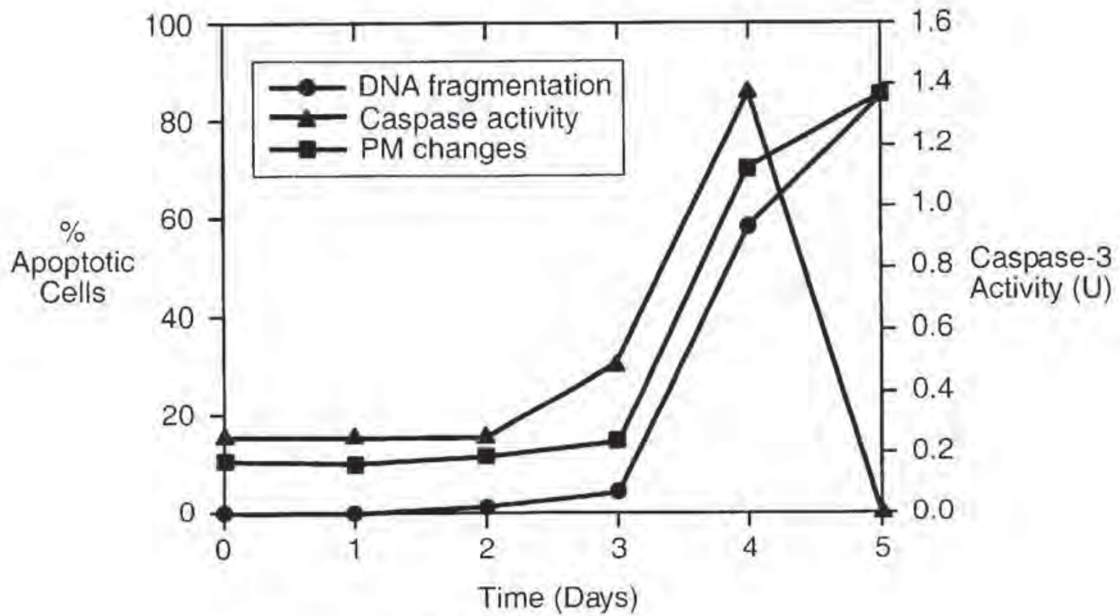

FIG._1B

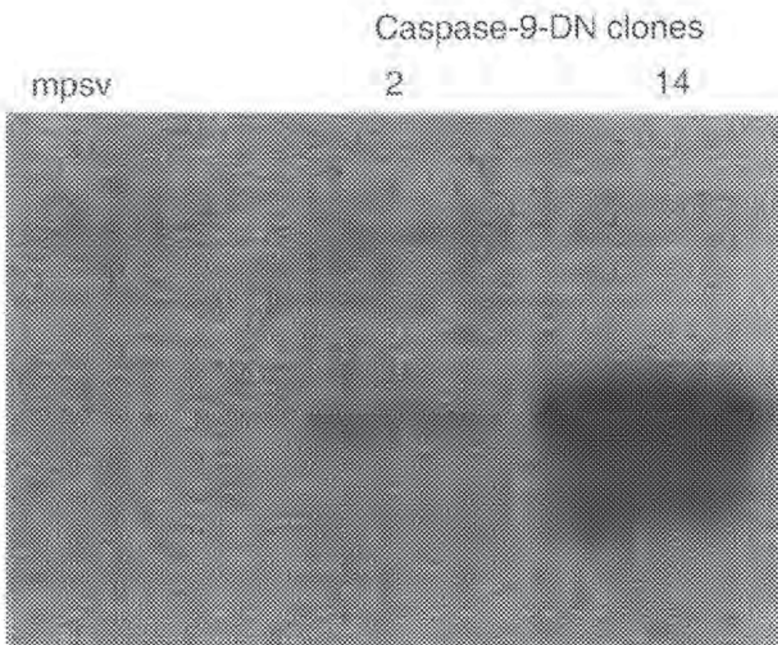

FIG._2

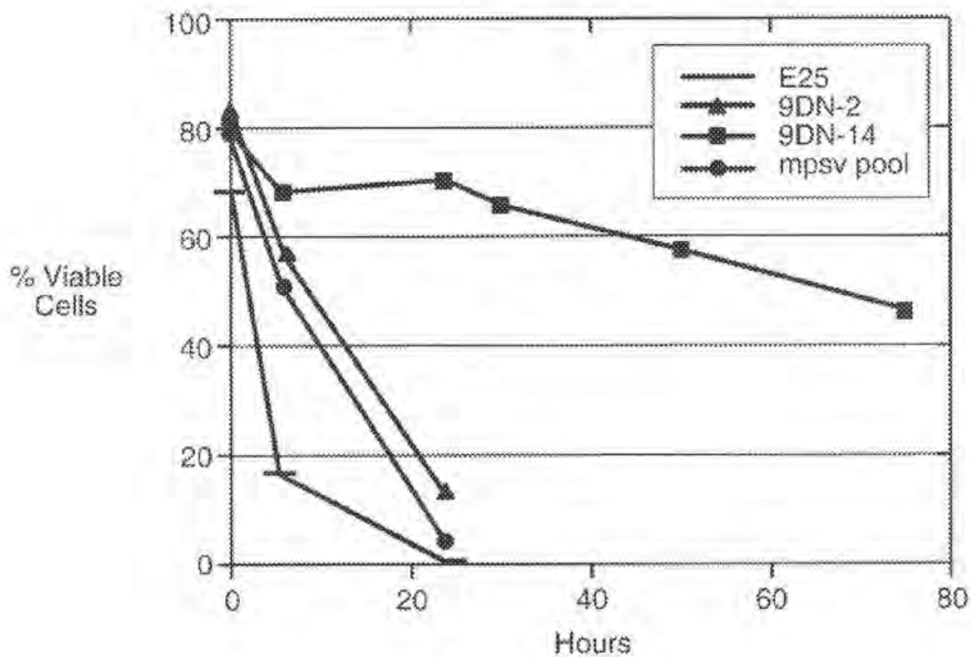

FIG._3

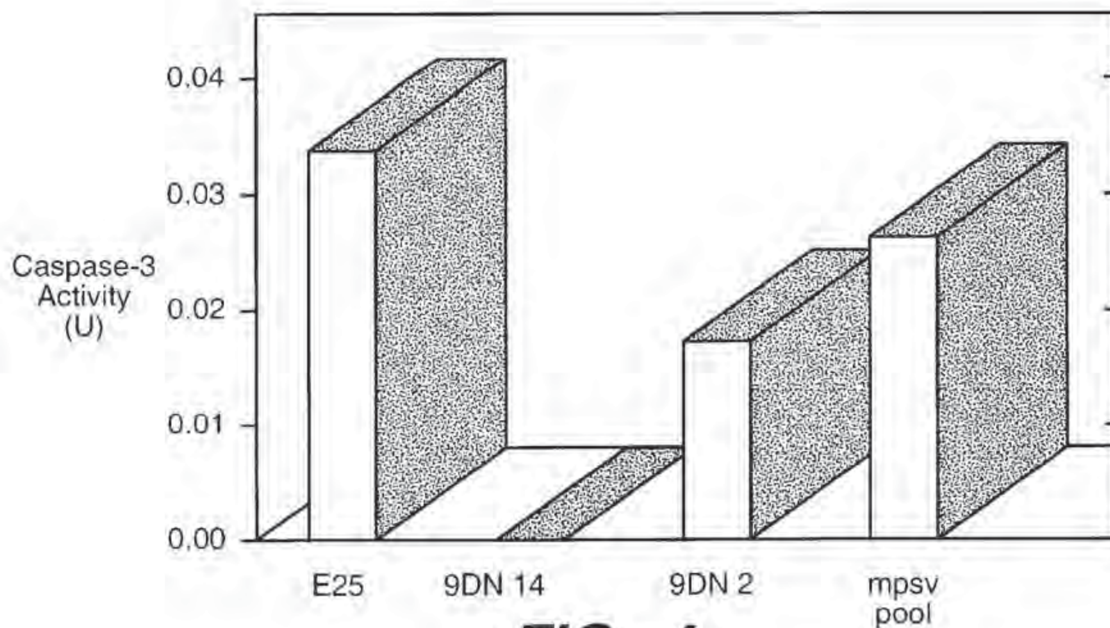

FIG._4

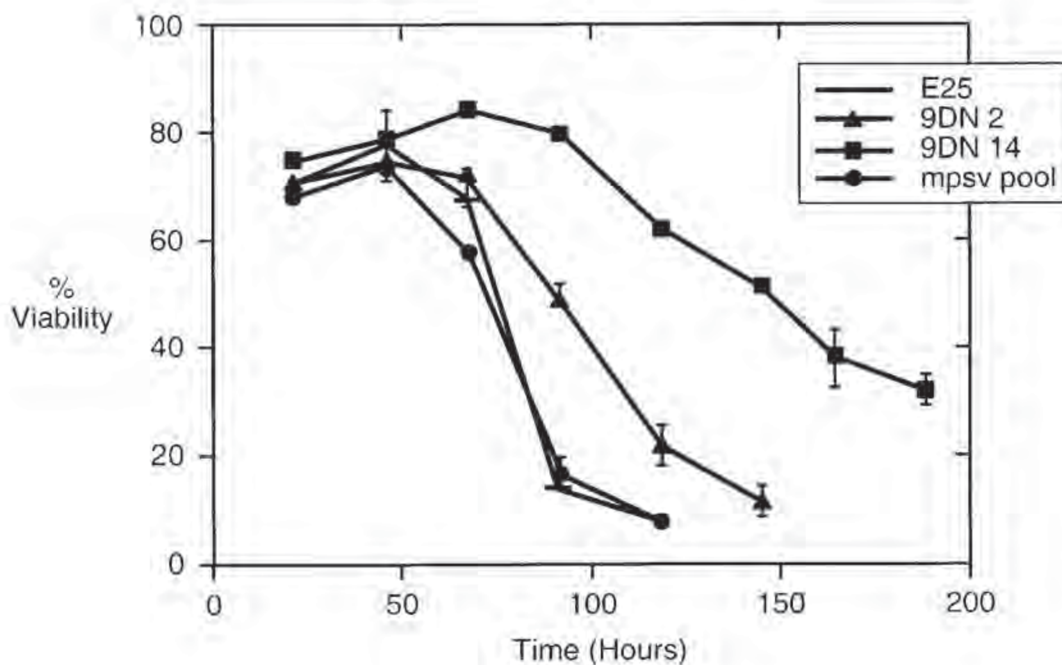

FIG._5

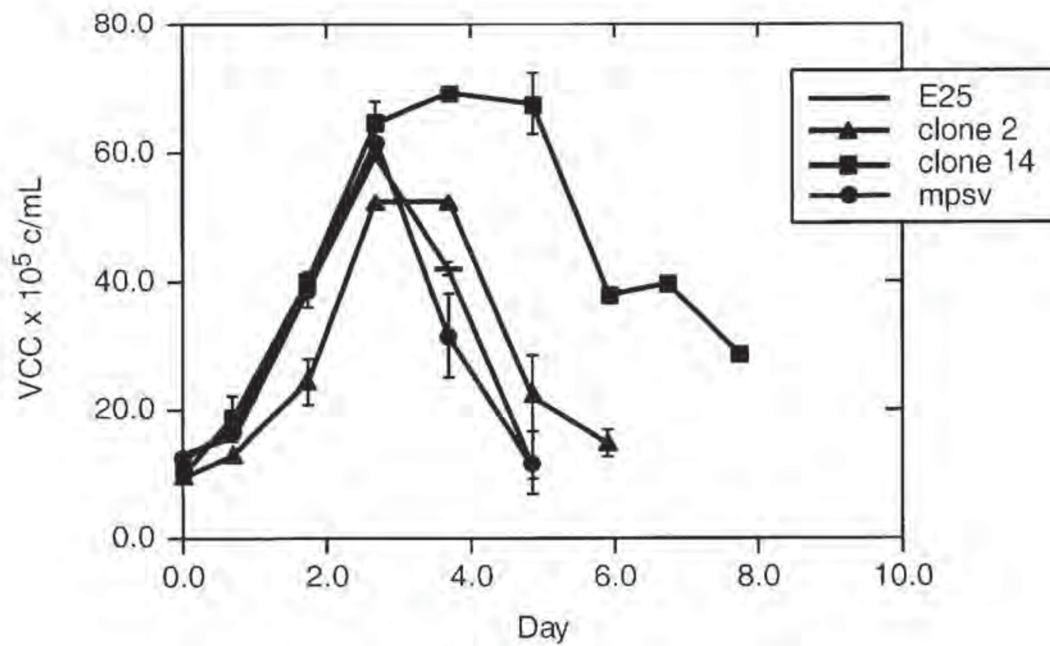

FIG._6

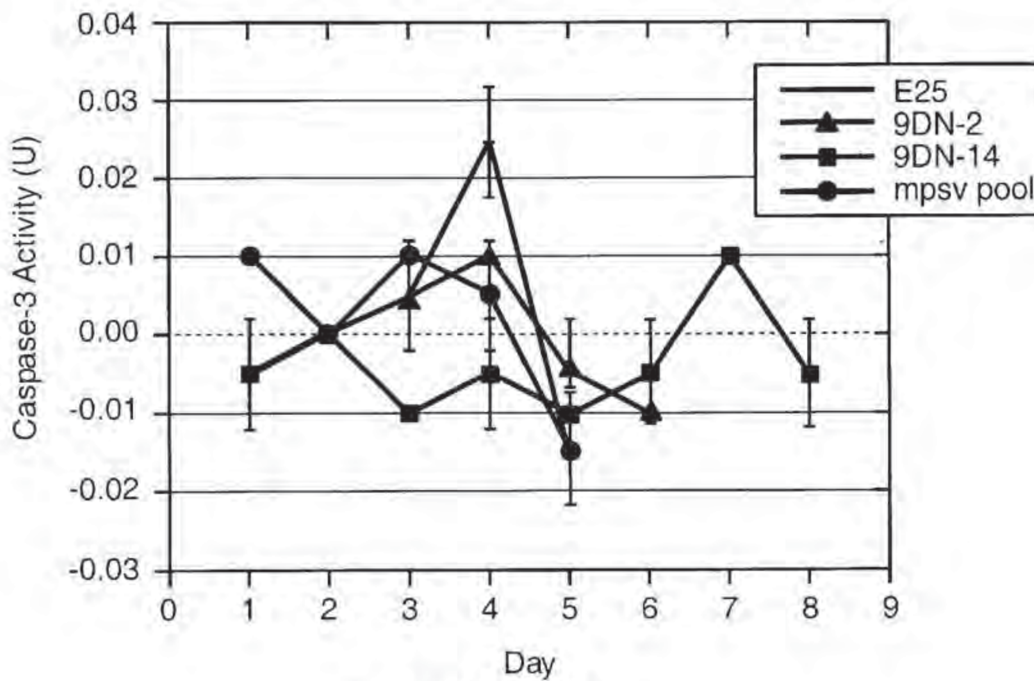

FIG._7

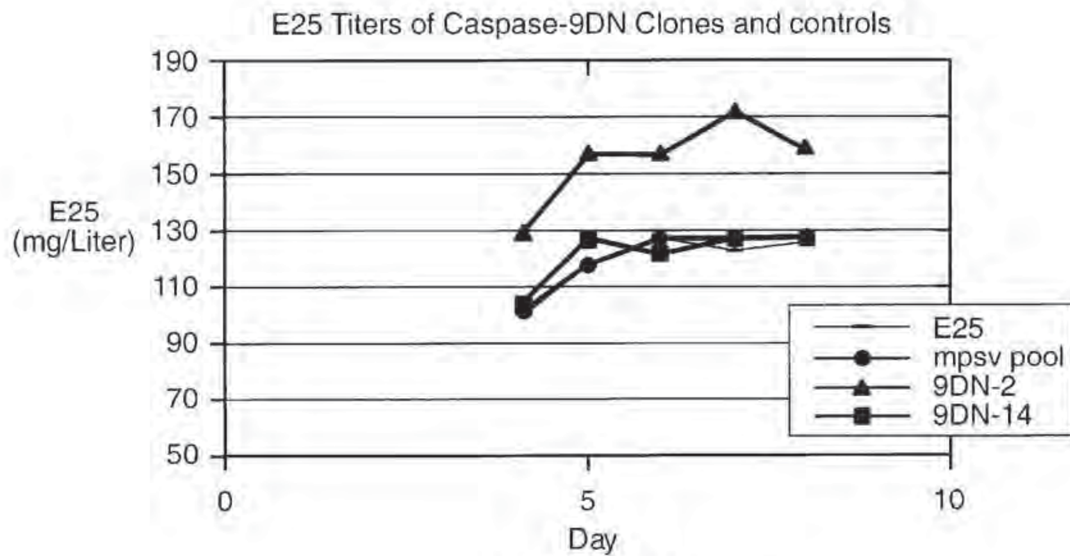

FIG._8

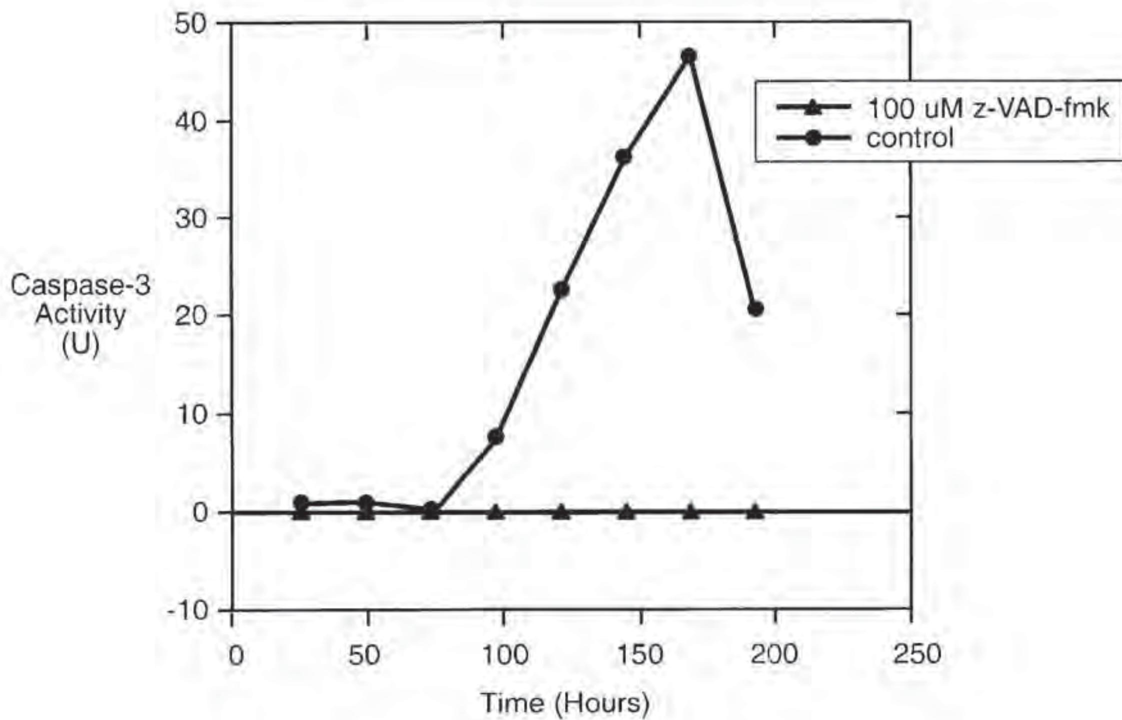

FIG._9

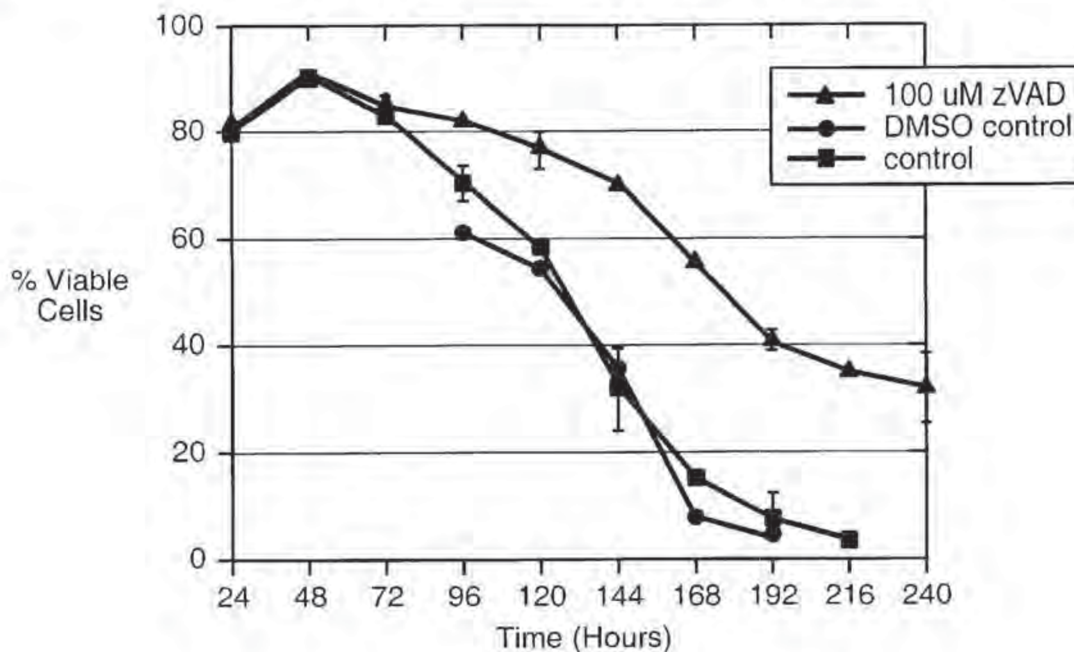

FIG._10

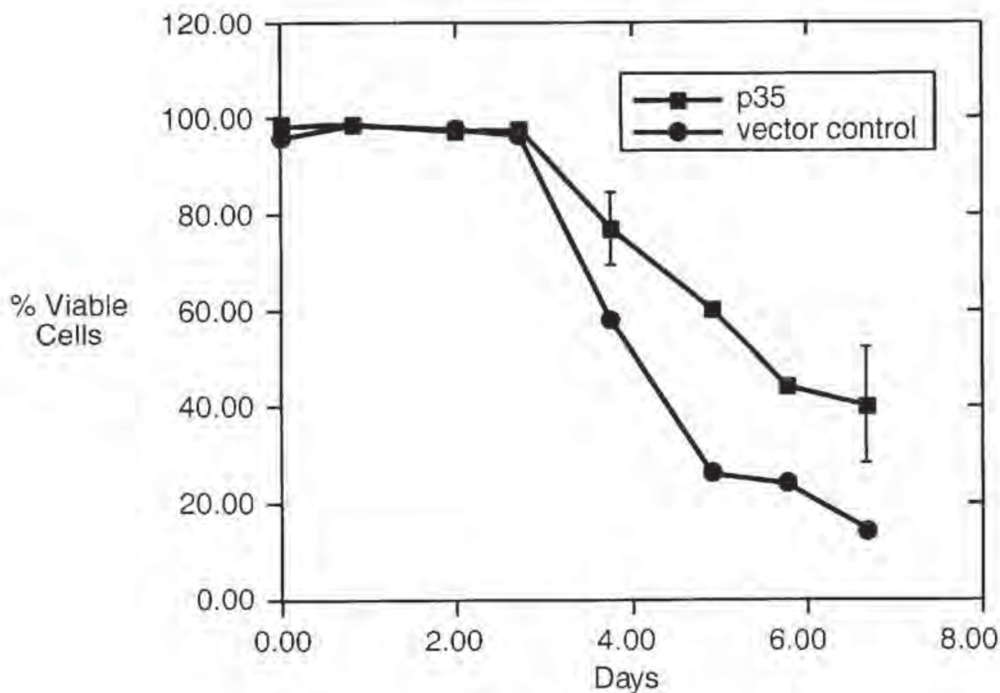

FIG._11

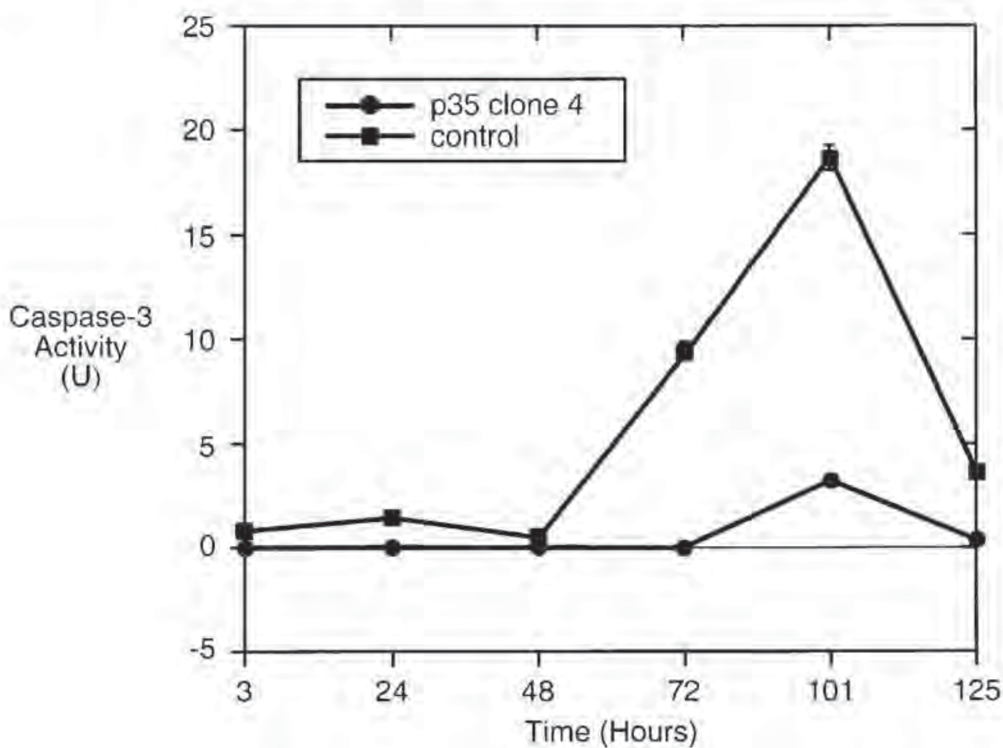

FIG._12

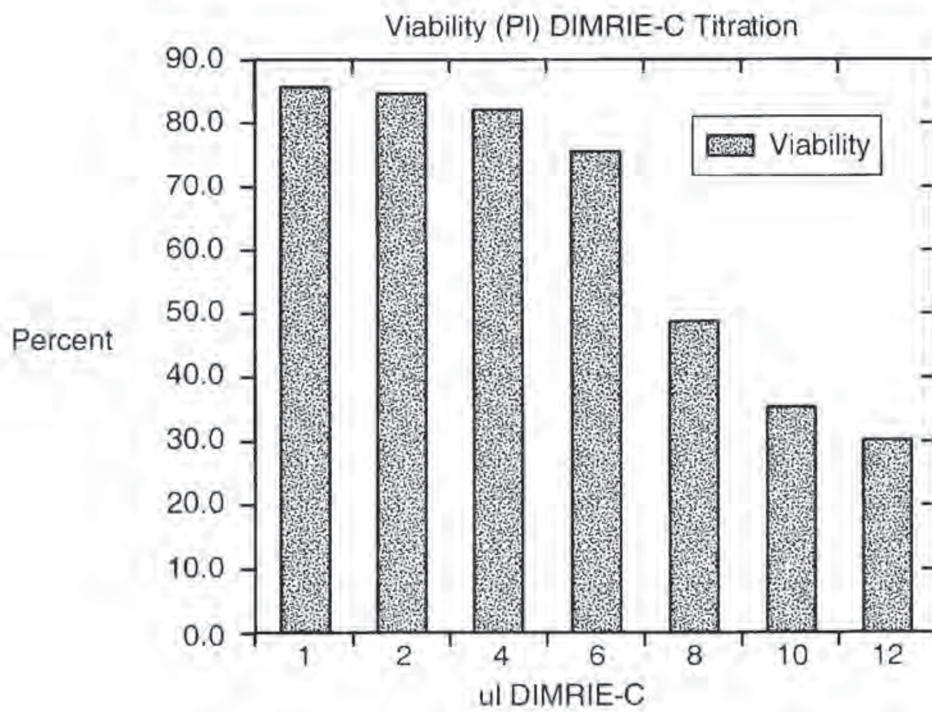

FIG._13

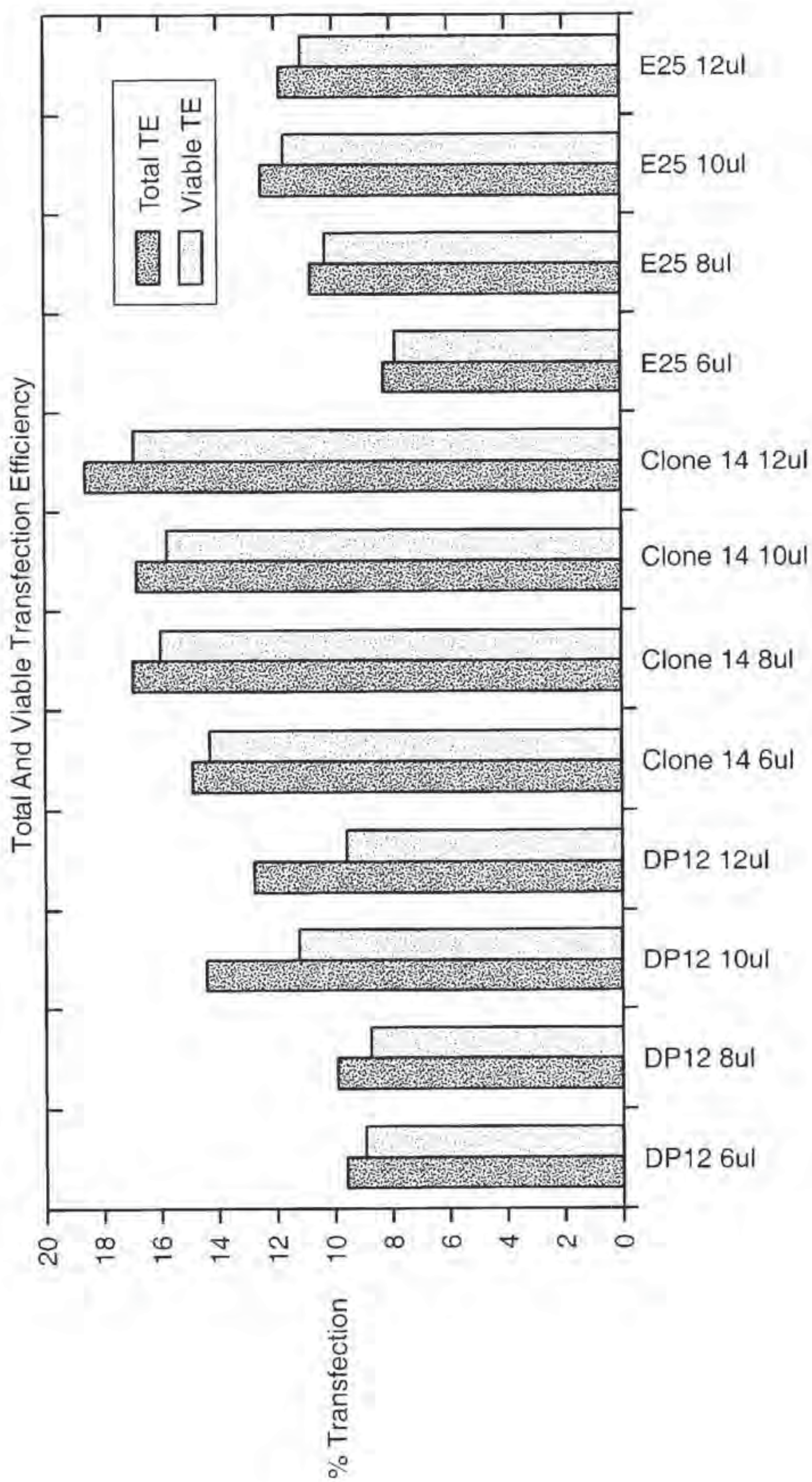

FIG._14

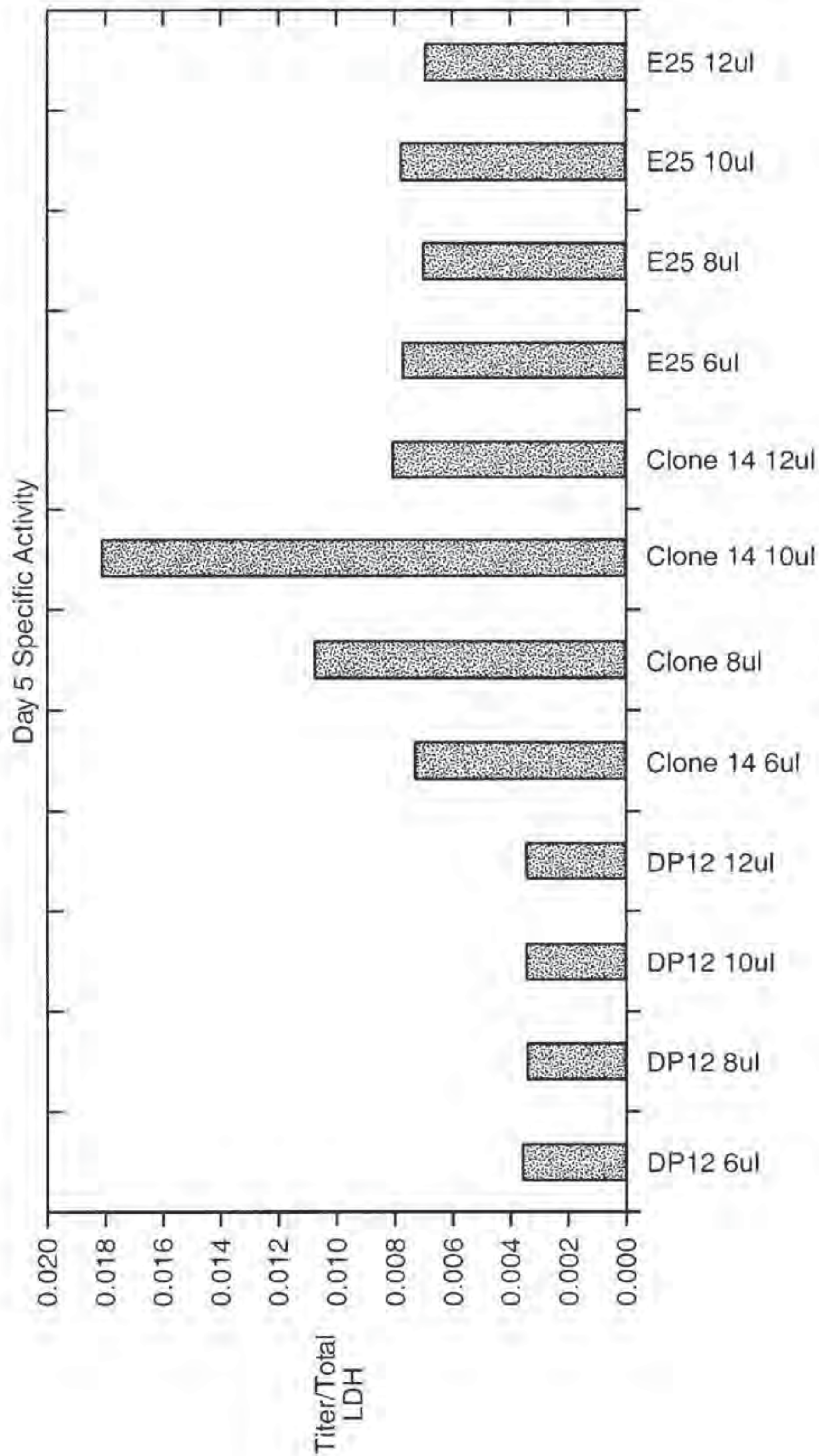

FIG._15

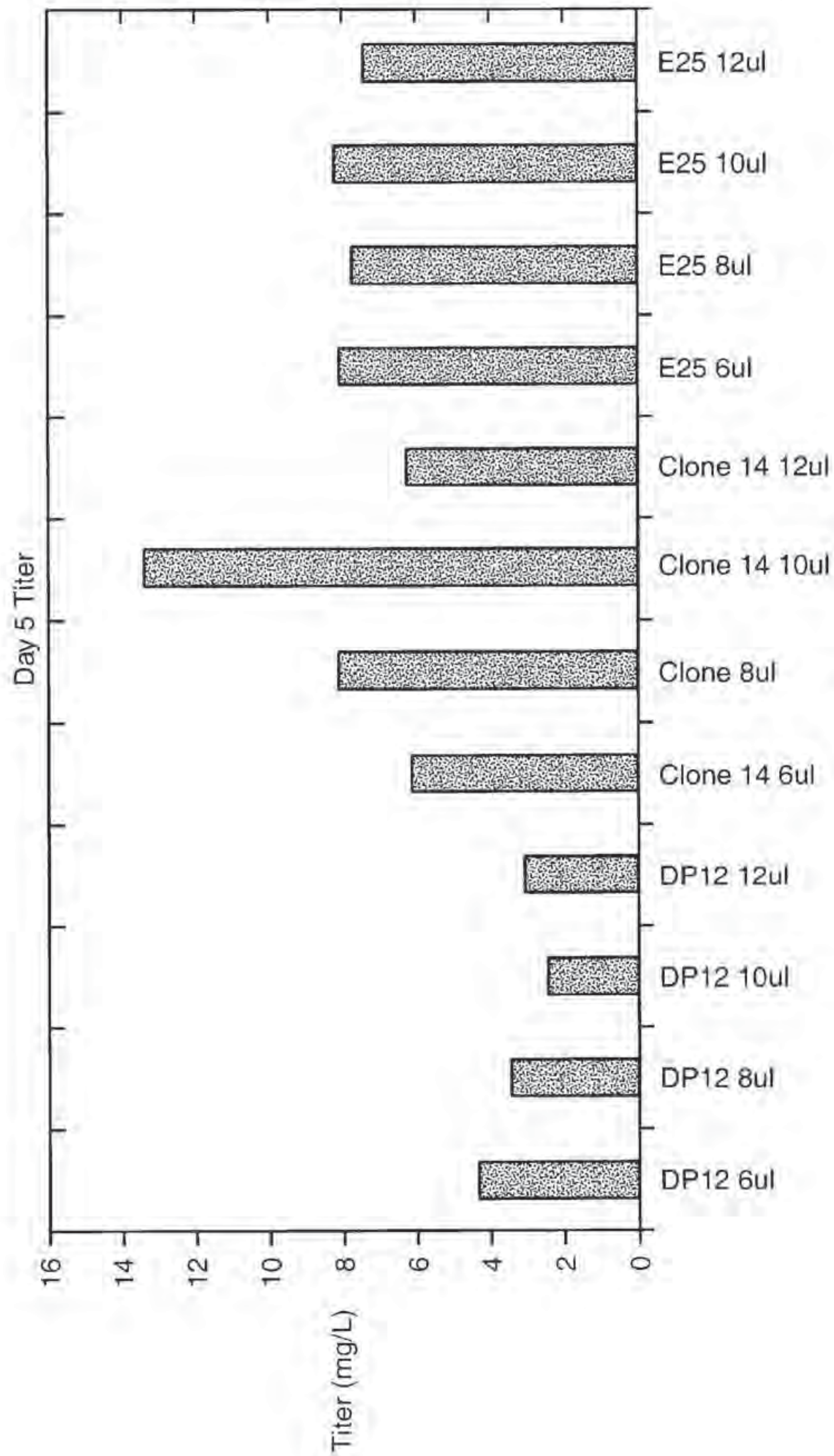

FIG._16

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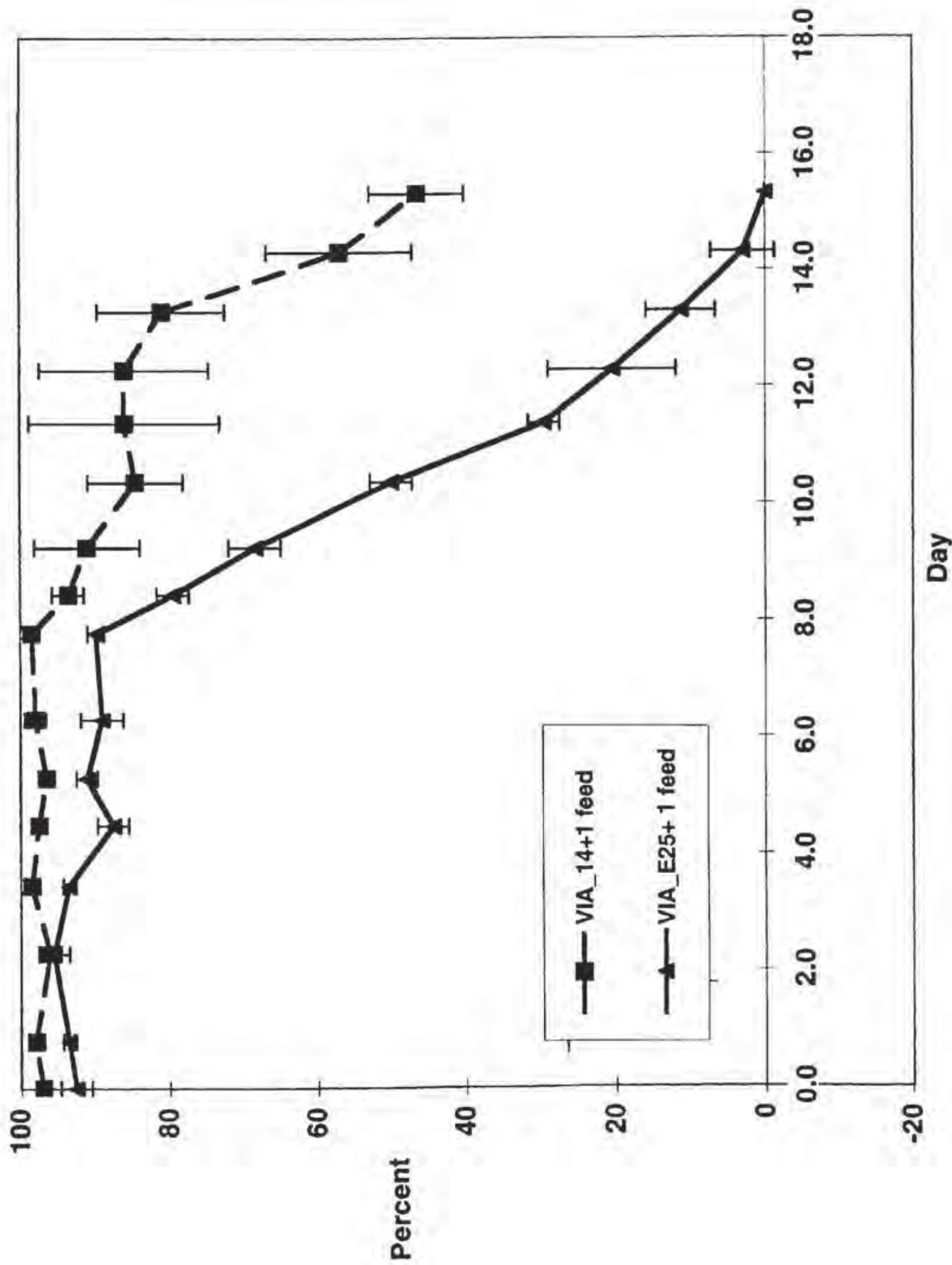

FIG._17

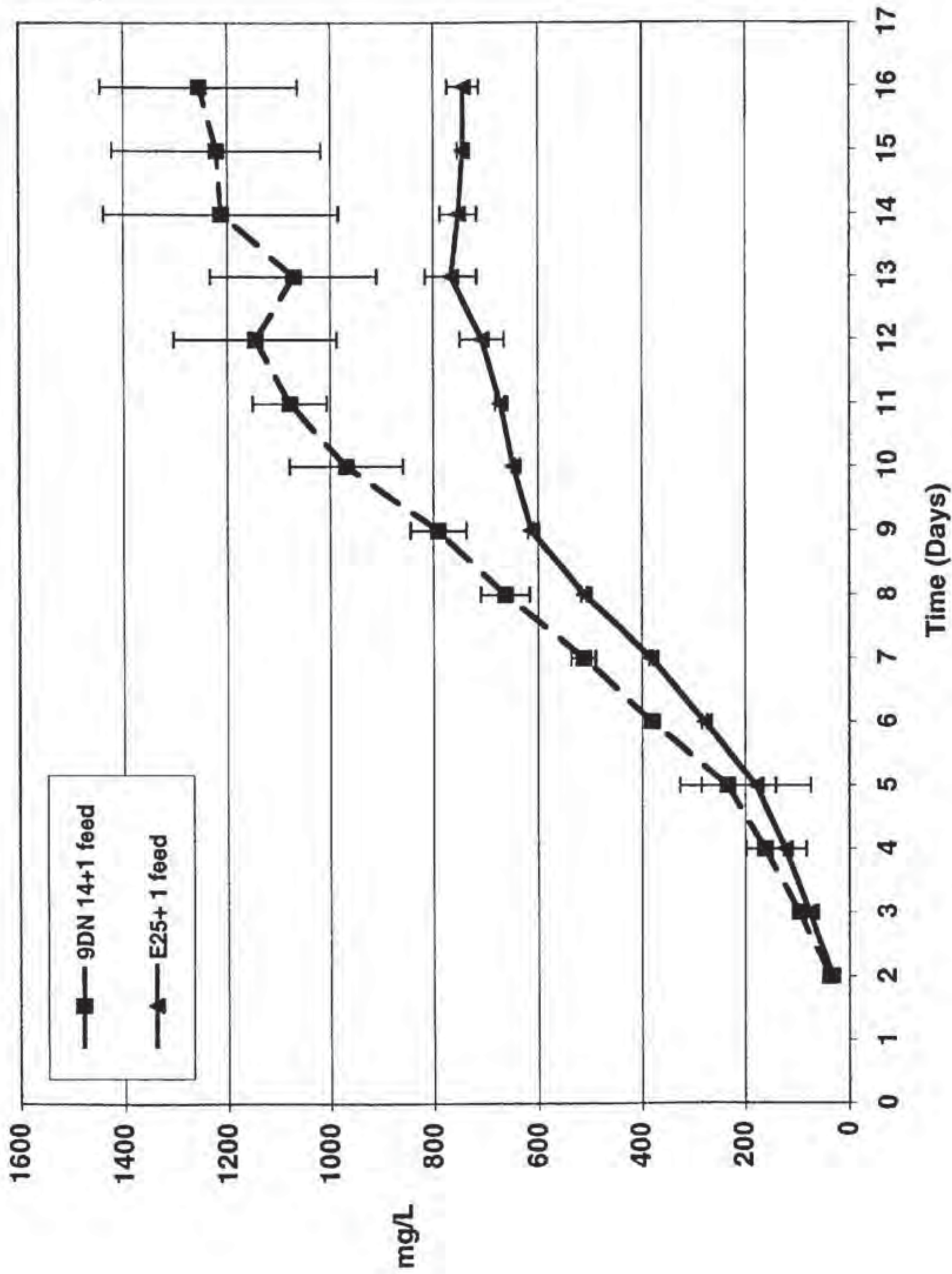

FIG._18

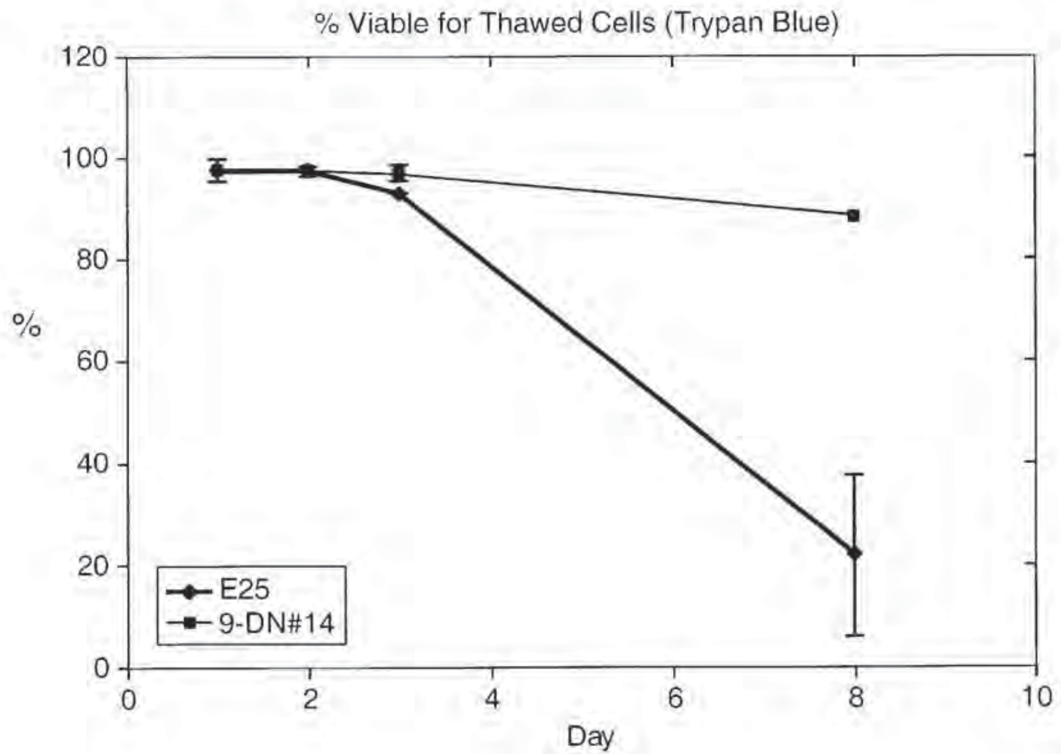

FIG._19

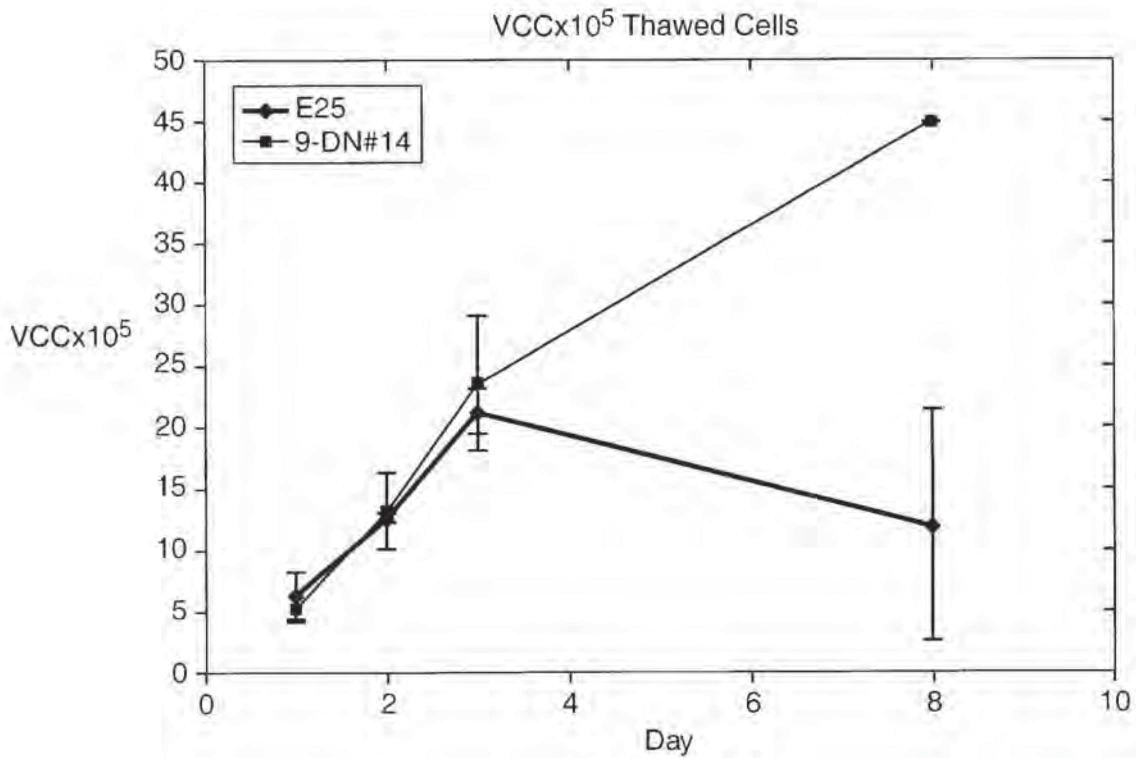

FIG._20

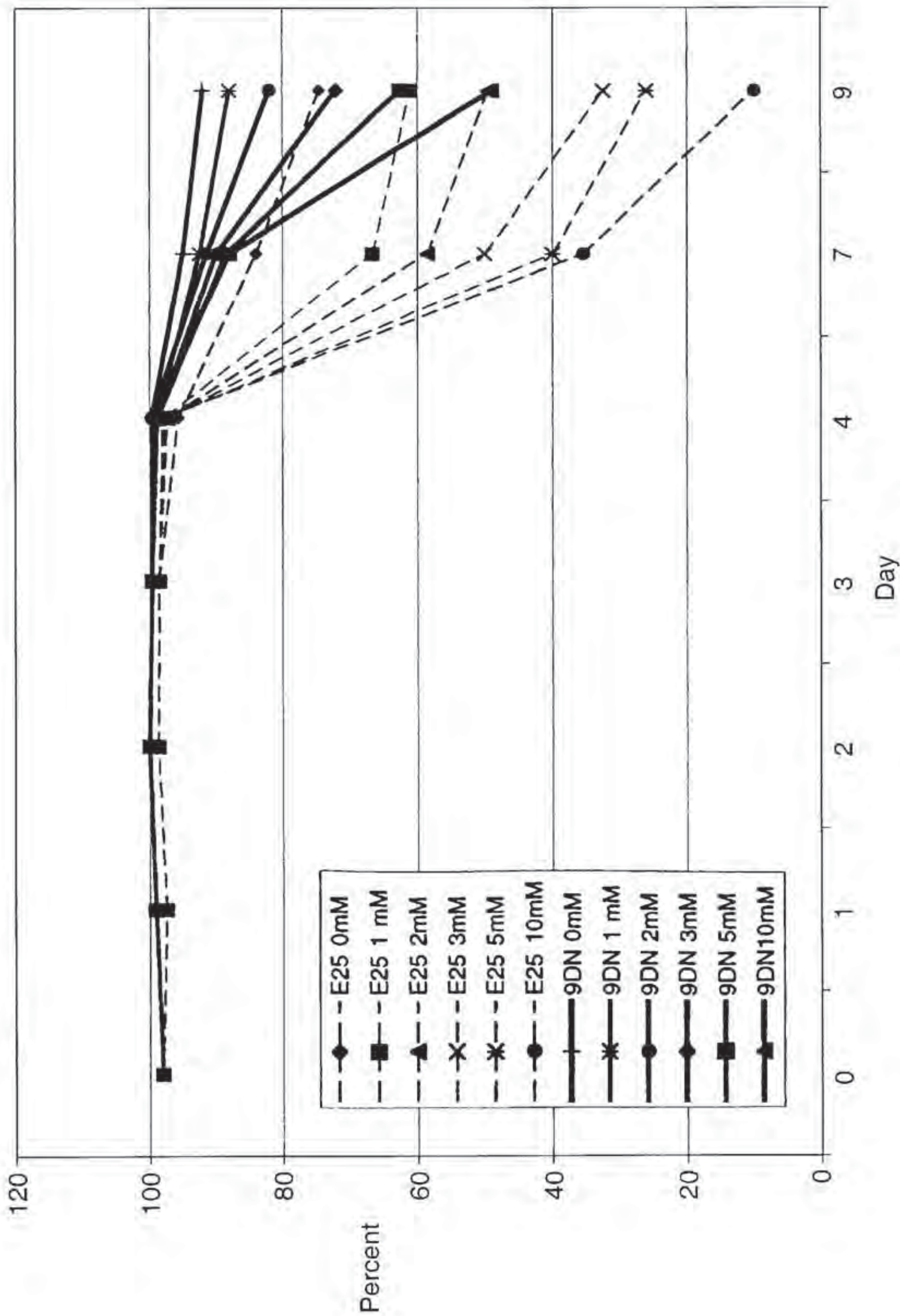

FIG._21

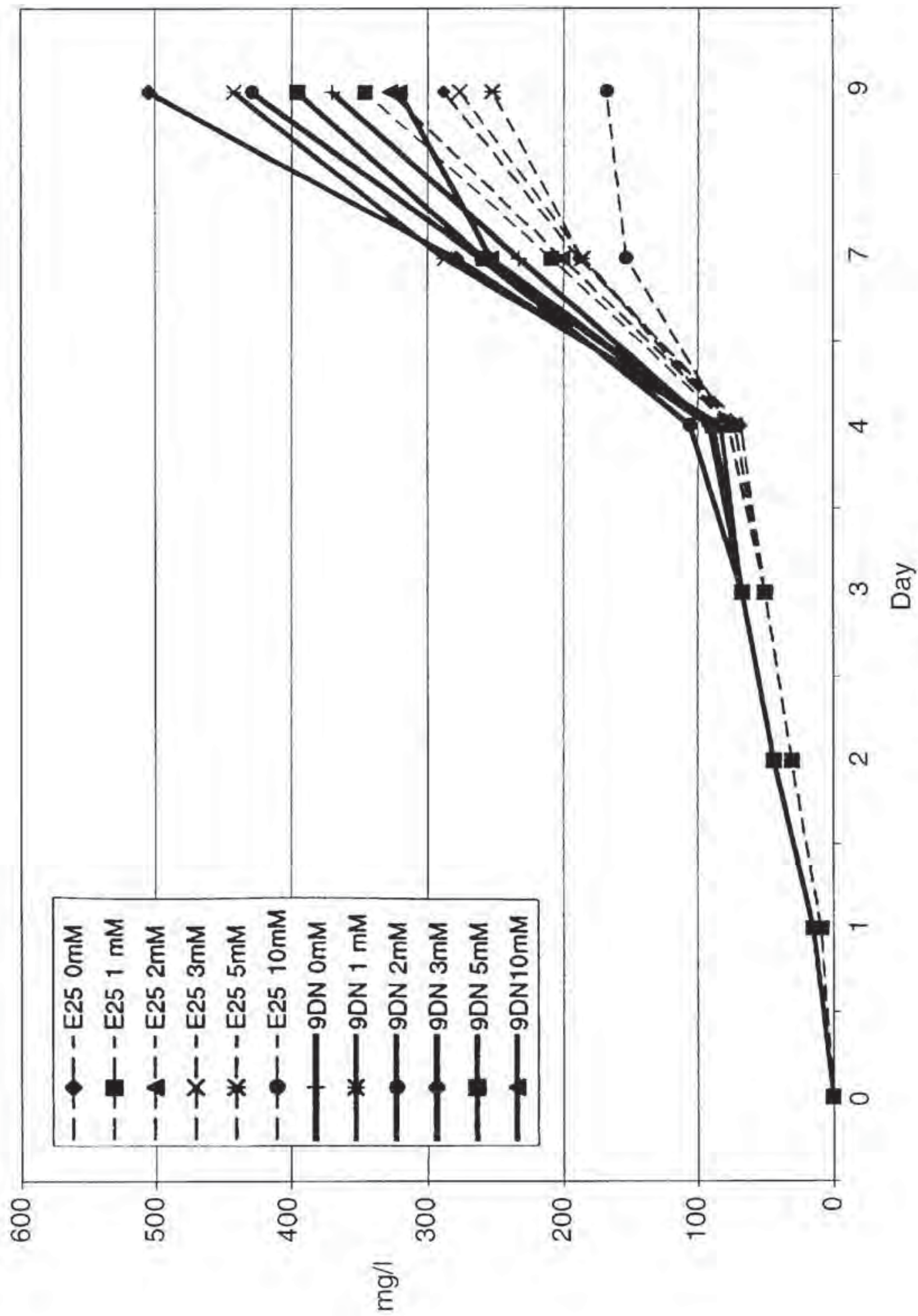

FIG._22

METHODS FOR MAKING RECOMBINANT PROTEINS USING APOPTOSIS INHIBITORS

RELATED APPLICATIONS

This is a non-provisional application claiming priority under Section 119(e) to provisional application no. 60/156,232, filed Sep. 27, 1999, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates generally to improved methods of making recombinant proteins using one or more apoptosis inhibitors.

BACKGROUND OF THE INVENTION

Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, e.g., Barr et al., *Bio/Technology,* 12:487–493 (1994); Steller et al., *Science,* 267:1445–1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., *Cell,* 66:233–243 (1991)].

Control of cell numbers in cell culture and bioreactors is also a balance between cell proliferation and cell death. There have been reports in the literature indicating cell death in bioreactors can be an apoptotic process [Suzuki E., et al., *Cytotechnology,* 23:55–59 (1997); Al-Rubeai, M. and Singh R. P, *Curr. Opin. Biotech,* 9:152–156 (1998)]. It has been described that the apoptotic process may be induced by nutrient deprivation [Franek F. and Chládkova-Šrámková K., *Cytotechnology,* 18:113–117 (1995); Mercille S. and Massie B., *Biotechnol. Bioeng.,* 44:1140–1154 (1994); Singh R. P., et al., *Biotechnol. Bioeng.,* 44:720–726 (1994)], serum deprivation [Singh R. P., et al., *Biotechnol. Bioeng.,* 44:720–726 (1994); Zanghi A., et al., *Biotech. Bioeng.,* 64:108–119 (1999)] or other controllable parameters of cell culture in bioreactors, but is not controlled fully because of bioreactor mechanics, a lack of full understanding of necessary culture parameters, or other undetermined causes.

As presently understood, the apoptosis or cell death program contains at least three important elements—activators, inhibitors, and effectors; in *C. elegans,* these elements are encoded respectively by three genes, Ced-4 4, Ced-9 and Ced-3 [Steller, *Science,* 267:1445 (1995); Chinnaiyan et al., *Science,* 275:1122–1126 (1997); Wang et al., *Cell,* 90:1–20 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, *Current Biology,* 6:555–62 (1996); Fraser and Evan, *Cell;* 85:781–784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF-KB [Tartaglia et al., *Cell,* 74:845–853 (1993); Hsu et al., *Cell,* 84:299–308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra; Nagata, *Cell,* 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the Drosophila protein, Reaper, and the mammalian proteins

referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, *Cell,* 81:479–482 (1995)].

Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signaling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., *Cell,* 81:505–512 (1995); Boldin et al., *J. Biol. Chem.,* 270:387–391 (1995); Hsu et al., supra; Chinnaiyan et al., *J. Biol. Chem.,* 271:4961–4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the Ced-3-related protease, MACH-alpha/FLICE (caspase 8), into the death signaling complex [Boldin et al., *Cell,* 85:803–815 (1996); Muzio et al., *Cell,* 85:817–827 (1996)]. MACH-alpha/FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1beta converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, ced-3, and to the mammalian IL-1-converting enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, crmA [Ray et al., *Cell,* 69:597–604 (1992); Tewari et al., *Cell,* 81:801–809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., *Nature,* 375:78–81 (1995); Tewari et al., *J. Biol. Chem.,* 270:3255–3260 (1995)].

As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF-KB [Tewari et al., *Curr. Op. Genet. Develop.,* 6:39–44 (1996)]. NF-KB is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., *Genes Develop.,* 9:2723–2735 (1996); Baldwin, *Ann. Rev. Immunol.,* 14:649–681 (1996)]. In its latent form, NF-KB is complexed with members of the IKB inhibitor family; upon inactivation of the IKB in response to certain stimuli, released NF-KB translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

For recent reviews of such signaling pathways, see, e.g., Ashkenazi et al., *Science,* 281:1305–1308 (1998); Nagata, *Cell,* 88:355–365 (1997).

To date, there have been conflicting reports as to the effects of caspase inhibitors and expression of anti-apoptotic genes on cultured recombinant cells. For instance, Murray et al., *Biotech. Bioeng.,* 51:298–304 (1996) describe that overexpression of bcl-2 in NSO myeloma cells failed to affect the decline phase characteristics of the cultured cells. Other investigators have found, in contrast, that bcl-2 can be effective in preventing different cell lines from death under cell-culture conditions [see, e.g., Itoh et al., *Biotechnol. Bioeng.,* 48:118–122 (1995); Mastrangelo et al., *TIBTECH,* 16:88–95 (1998); Simpson et al., *Biotechnol. Bioeng.,* 54:1–16 (1997); Singh et al., *Biotechnol. Bioeng.,* 52:166–175 (1996)]. Goswami et al., *Biotechnol. Bioeng.,* 62:632–640 (1999) report that they found that the caspase inhibitor, z-VAD-fmk, was unable to substantially extend the life of a serum-free culture of CHO cells.

SUMMARY OF THE INVENTION

The present invention is based on Applicants' findings that employing one or more apoptosis inhibitor(s) in recom-

binant cell culturing and protein production can markedly reduce apoptosis in the cell culture and improve recombinant protein production techniques. The methods disclosed in present application are useful, for example, in prolonging cell viability in cell cultures or improving or enhancing yield of the recombinant proteins from the cell cultures. Further improvements provided by the invention are described in detail below.

In one embodiment, the invention provides a method of making recombinant proteins using one or more apoptosis inhibitors. The method includes the steps of (a) providing a vector comprising a gene encoding an apoptosis inhibitor, (b) providing a vector comprising a gene encoding a protein of interest, (c) providing a host cell, (d) transforming or transfecting the host cell with the vectors referred to in steps (a) and (b), (e) providing cell culture media, (f) culturing the transformed or transfected host cell(s) in the culture media under conditions sufficient to express the protein of interest and the apoptosis inhibitor, and (g) recovering or purifying the protein of interest from the host cells and/or the cell culture media. Optionally, the method further includes the step of admixing an additional apoptosis inhibitor into the culture media. In the method, the respective genes encoding the apoptosis inhibitor and the protein of interest may be inserted into a single vector (e.g., co-transfected in a single vector), or alternatively, be inserted into two separate vectors. Preferably, the respective genes encoding the apoptosis inhibitor and the protein of interest are inserted into two separate vectors, each vector having a different type of selection marker from the other vector. Optionally, the method provides for transient expression of the protein of interest and stable or transient expression of the apoptosis inhibitor. Optionally, the gene encoding the apoptosis inhibitor comprises a gene encoding the caspase-9-DN protein or baculovirus p35.

In another embodiment, the method includes the steps of (a) providing a vector comprising a gene encoding a protein of interest, (b) providing a host cell comprising DNA encoding an apoptosis inhibitor, (c) transforming or transfecting the host cell(s) with the vector referred to in step (a), (d) providing cell culture media, (e) culturing the transformed or transfected host cell(s) in the culture media under conditions sufficient to express the protein of interest and the apoptosis inhibitor, and (f) recovering or purifying the protein of interest from the host cells and/or cell culture media. Optionally, the gene encoding the apoptosis inhibitor may be stably integrated into the genome of the host cell. Optionally, the method includes the further step of admixing an additional apoptosis inhibitor molecule into the culture media. Optionally, the method provides for transient expression of the protein of interest and stable or transient expression of the apoptosis inhibitor.

In another embodiment, the method includes the steps of (a) providing a vector comprising a gene encoding a protein of interest, (b) providing a host cell, (c) transforming or transfecting the host cell with the vector referred to in step (a), (d) providing cell culture media, (e) providing an apoptosis inhibitor, (f) admixing the apoptosis inhibitor into the culture media, (g) culturing the host cell(s) in the culture media under conditions sufficient to express the protein of interest, and (h) recovering or purifying the protein of interest from the host cells and/or the cell culture media. Optionally, the method provides for transient expression of the protein of interest.

In another embodiment, the method includes the steps of (a) providing a vector comprising a gene encoding an apoptosis inhibitor, (b) providing a vector comprising a gene encoding a protein of interest, (c) providing a host cell, (d) transforming or transfecting the host cell with the vectors referred to in steps (a) and (b), (e) providing cell culture media, (f) culturing the transformed or transfected host cell(s) in the culture media under conditions sufficient to express the protein of interest and the apoptosis inhibitor, and (g) freezing and subsequently thawing the host cells and/or the cell culture media. Optionally, the method further includes the step of admixing an additional apoptosis inhibitor into the culture media in steps (e) or (f). In the method, the respective genes encoding the apoptosis inhibitor and the protein of interest may be inserted into a single vector, or alternatively, be inserted into two separate vectors. Preferably, the respective genes encoding the apoptosis inhibitor and the protein of interest are inserted into two separate vectors, each vector having a different type of selection marker from the other vector. Optionally, the method provides for transient expression of the protein of interest and stable or transient expression of the apoptosis inhibitor.

In another embodiment, the method includes the steps of (a) providing a vector comprising a gene encoding a protein of interest, (b) providing a host cell comprising DNA encoding an apoptosis inhibitor, (c) transforming or transfecting the host cell(s) with the vector referred to in step (a), (d) providing cell culture media, (e) culturing the transformed or transfected host cell(s) in the culture media under conditions sufficient to express the protein of interest and the apoptosis inhibitor, and (f) freezing and subsequently thawing the host cells and/or cell culture media. Optionally, the gene encoding the apoptosis inhibitor may be stably integrated into the genome of the host cell. Optionally, the method includes the further step of admixing an additional apoptosis inhibitor molecule into the culture media. Optionally, the method provides for transient expression of the protein of interest and stable or transient expression of the apoptosis inhibitor.

In another embodiment, the method includes the steps of (a) providing a vector comprising a gene encoding a protein of interest, (b) providing a host cell, (c) transforming or transfecting the host cell with the vector referred to in step (a), (d) providing cell culture media, (e) providing an apoptosis inhibitor, (f) admixing the apoptosis inhibitor into the culture media, (g) culturing the host cell(s) in the culture media under conditions sufficient to express the protein of interest, and (h) freezing and subsequently thawing the host cells and/or the cell culture media. Optionally, the method provides for transient expression of the protein of interest.

In a still further embodiment, the invention provides for improved transfection methods wherein use of one or more apoptosis inhibitor(s) and increased concentrations of transfection reagent can be employed to increase transfection efficiency.

In an even further embodiment, the invention provides a protein of interest produced in accordance with any of the methods described herein. The protein of interest may comprise a mammalian protein or non-mammalian protein, and may optionally comprise a receptor or a ligand. In one embodiment of the invention, the protein of interest will comprise a protein which itself is capable of inducing apoptosis in mammalian or non-mammalian cells in vitro or in vivo, such as Apo-2 ligand/TRAIL, Fas ligand, or TNF-alpha.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows a graph illustrating cell viability of CHO cells grown in a 2 liter bioreactor. The data show that the

cells grown in bioreactors may begin to lose viability as early as day 3, often followed by a dramatic drop in viability on the following day(s).

FIG. **1B** shows a graph illustrating the results of three apoptosis assays performed on the CHO cells (referred to in FIG. **1A** and Example 1): caspase-3 activation, DNA fragmentation and annexin/PI binding (plasma membrane ("PM") changes). Activation of caspases was first detected on day 3, the day that drop in viability was detected (FIG. **1A**).

FIG. **2** shows a Western blot analysis of lysates from caspase-9-DN transfected clones. A clone transfected with a mpsv vector (alone) was used as a control. The blot was probed with rabbit anti-caspase-9 antiserum (Pharmingen) and developed using chemiluminescence.

FIG. **3** shows the results of an assay wherein caspase-9-DN clones 2 and 14, as well as controls (E25 untransfected cells and mpsv vector transfected cells) were incubated with an apoptosis inducer, staurosporine (1 micromolar). Samples were taken and cells were analyzed for the % of viable cells.

FIG. **4** shows an analysis of caspase-3 activity or cell samples taken at 24 hours post-induction with 1 micromolar staurosporine.

FIGS. **5–8** show assay results of caspase-9-DN expressing clones 2 and 14, as well as controls, scaled up and seeded at 1 million cells/ml in a 2 liter bioreactor. Samples were taken daily and were analyzed for viability (FIG. **5**), viable cell count (FIG. **6**), activity of caspase-3 (FIG. **7**) and the concentration of the protein of interest (E25 antibody) secreted into the medium (FIG. **8**).

FIGS. **9–10** show assay results of CHO cells seeded in 60 mm dishes and exposed to caspase inhibitor, z-VAD-fmk (added to the cell culture at 100 micromolar concentration, 48 hours after seeding). The z-VAD-fmk inhibitor was added to the culture every 24 hours thereafter. Samples were taken every day and analyzed for caspase-3 activity (FIG. **9**) and the % viable cells (FIG. **10**).

FIGS. **11–12** show assay results of a Baculovirus p35 expressing clone grown in a 2 liter bioreactor and assayed daily for cell viability (FIG. **11**) and caspase-3 activity (FIG. **12**). The control is a clone transfected with a vector, cpc.

FIG. **13** shows a bar diagram of the effects of various concentrations of the transfection reagent, DMRIE-C, on cell viability.

FIG. **14** shows a comparison of total and viable transfection efficiencies obtained for caspase-9-DN clone 14 and controls, CHO DP12 cells and E25 antibody expressing CHO DP12 cells.

FIG. **15** shows a comparison of the specific productivity (as measured in Dnase titer/total LDH) obtained for caspase-9-DN clone 14 and controls, CHO DP12 cells and E25 antibody expressing CHO DP12 cells.

FIG. **16** shows a comparison of the DNase titer obtained for caspase-9-DN clone 14 and controls, CHO DP12 cells and E25 antibody expressing CHO DP12 cells.

FIGS. **17** and **18** show viability and titers of caspase-9-DN and E25 control grown in 2 liter bioreactors with temperature shift, concentrated medium and a feed.

FIGS. **19** and **20** show viability and viable cell count of cultures of E25 control and caspase-9-DN clone 14 seeded into spinners from frozen vials. Data were obtained by trypan blue exclusion.

FIGS. **21** and **22** show viability and E25 titers of cultures of E25 control cells and caspase-9-DN clone 14 upon induction of expression by butyrate.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The term "apoptosis inhibitor" is used herein to refer to a molecule or substance whose expression or presence in an in vitro cell culture provides a reduction or inhibition of apoptosis in the cultured cells, or provides resistance of the cultured cells to apoptotic stimuli. The apoptosis inhibitor may comprise a protein or protein-like molecule, or an organic or inorganic molecule. The apoptosis inhibitor may be present (and/or function) intracellularly, extracellularly, or at the cell surface (membrane) of the cultured cells. Particular apoptosis inhibitors contemplated by the present invention include, but are not limited to, the caspase-9 dominant negative (caspase-9-DN) mutant, bcl-2, baculovirus p35, caspase-9S (Seol,D. W. et al., *J. Biol. Chem.*, 274, 2072–2076 (1999)), crmA, z-VAD-fmk, z-DEVD-fmk, B-D-fmk, and z-YVAD-fmk, and variants therof. Preferably, the apoptosis inhibitor is one which acts upon one or more caspases located downstream in the intracellular cell death pathway of the cell, such as caspase-3. Optionally, the apoptosis inhibitor will, in an effective amount, decrease or reduce apoptosis in a cell culture by at least 50%, preferably, by at least 75%, more preferably, by at least 85%, and even more preferably, by at least 95%, as compared to a control cell culture which contains no such apoptosis inhibitor. Apoptosis or apoptotic activity in such cell cultures can be measured and determined using assays such as described herein. Optionally, the apoptosis inhibitor, in an effective amount, will enhance or increase yield of the recombinant protein of interest by at least 1-fold, and preferably by at least 2-fold, as compared to a control cell culture which contains no such apoptosis inhibitor. Optionally, the apoptosis inhibitor, in an effective amount, will enhance or increase transfection efficiency in transient transfections, preferably by at least 1-fold and more preferably, by at least 2-fold, as compared to a control cell culture which contains no such apoptosis inhibitor.

The term "protein of interest" refers to any protein which may be useful for research, diagnostic or therapeutic purposes. The protein of interest may comprise a mammalian protein or non-mammalian protein, and may optionally comprise a receptor or a ligand. Exemplary proteins of interest include, but are not limited to, molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone;. thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; members of the TNF and TNF receptor (TNFR) family, like tumor necrosis factor-alpha and -beta, CD40 ligand, Apo-2 ligand/TRAIL, DR4, DR5, DcR1, DcR2, DcR3, OPG, Fas ligand; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA),

such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TG-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; thrombopoietin (TPO); interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope, gp120; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and variants and/or fragments of any of the above-listed polypeptides; as well as antibodies against various protein antigens like CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150.95, VLA-4, ICAM-1, VCAM and αv/β3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C; an Apo-2L receptor such as Apo-2 (DR5), DR4, DcR1, DcR2, DcR3; and variants and/or fragments of the above-identified antibodies etc. In one embodiment of the invention, a protein of interest will comprise a protein which itself is capable of inducing apoptosis in mammalian or non-mammalian cells in vitro or in vivo, such as Apo-2 ligand/TRAIL, Fas ligand, or TNF-alpha.

"Isolated," when used to describe the various proteins of interest disclosed herein, means protein that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with research, diagnostic or therapeutic uses for the protein of interest, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the protein will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein in situ within recombinant cells, since at least one component of the protein of interest's natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordace with conventional practice.

As used herein, the expressions "cell", "cell line", and "cell culture" are used interchangeably and all such designations include progeny. Thus, the terms "transformants" and "transfectants" include the primary subject cell and cultures derived therefrom without regard for the number of transfers.

"Growth phase" of the cell culture refers to the period of exponential cell growth (the log phase) where cells are generally rapidly dividing. During this phase, cells are cultured for a period of time, usually between 1–4 days, and under such conditions that cell growth is maximized. The determination of the growth cycle for the host cell can be determined for the particular host cell envisioned without undue experimentation. "Period of time and under such conditions that cell growth is maximized" and the like, refer to those culture conditions that, for a particular cell line, are determined to be optimal for cell growth and division. During the growth phase, cells are cultured in nutrient medium containing the necessary additives generally at about 30–40° C., preferably about 37° C., in a humidified, controlled atmosphere, such that optimal growth is achieved for the particular cell line. Cells are maintained in the growth phase for a period of about between one and four days, usually between two to three days.

"Transition phase" of the cell culture refers to the period of time during which culture conditions for the production phase are engaged. During the transition phase environmental factors such as pH, ion concentration, and temperature may shifted from growth conditions to production conditions.

"Production phase" of the cell culture refers to the period of time during which cell growth has reached a plateau. During the production phase, logarithmic cell growth has ended and protein production is primary. During this period of time the medium is generally supplemented to support continued protein production and to achieve the desired protein product.

The term "expression" or "expresses" is used herein to refer to transcription and translation occurring within a host cell. The level of expression of a product gene in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present in the cell or the amount of the protein encoded by the product gene that is produced by the cell. For example, mRNA transcribed from a product gene is desirably quantitated by northern hybridization. See, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, pp. 7.3–7.57 (Cold Spring Harbor Laboratory Press, 1989). Protein encoded by a product gene can be quantitated either by assaying for the biological activity of the protein or by employing assays that are

independent of such activity, such as western blotting or radioimmunoassay using antibodies that are capable of reacting with the protein. See, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, pp. 18.1–18.88 (Cold Spring Harbor Laboratory Press, 1989).

The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammalian or non-mammalian cells that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, activation of caspase(s), segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and measured, for instance, by cell viability assays, FACS analysis, annexin V binding, or DNA electrophoresis such as is known in the art and described further herein.

II. The Methods of the Invention

Cells grown in cell culture may begin to lose viability within days of initiating the culture. Loss of cell viability can particularly be problematic when culturing cells in relatively large, batch scale cultures or bioreactors. For instance, CHO cells grown in batch culture can begin to lose cell viability as early as Day 4 after which a rapid decline in viability can continue until the culture is terminated. The mechanism by which such cultured cells die may be either through necrosis or apoptosis. Using TUNEL and Annexin/PI binding assays, Applicants discovered that approximately 80% of some CHO cells grown in batch culture may die by apoptosis rather than through necrosis. As described herein, Applicants have surprisingly found methods which allow a marked reduction of such apoptosis.

The methods disclosed in the present application have a variety of applications and improvements for recombinant protein production. First, by prolonging host cell viability in culture (and during fermentation), one skilled in the art can increase production and yield of the protein of interest. This can improve the efficiency of the cell culture run and result in marked cost savings. Further, Applicants have found the use of one or more apoptosis inhibitors in the methods of the invention may protect against potential adverse effects of agents like butyrate or TSA included in the cell culture. Also, the methods herein can enhance quality of the expressed and recovered protein of interest. The quality of the expressed and recovered protein of interest may be evaluated using techniques known in the art, such as SDS-PAGE, etc. The occurrence of cell death in recombinant cell cultures oftentimes results in the release of various active proteins from the dying cells, such as proteases [Lao, M., et al., *Cytotechnology,* 22: 43–52 (1996); Teige, M., et al., *J. Biotechnol.,* 34:101–105 (1994)], glycosidases such as sialidase or β-galactosidase [Gramer M. J. and Goochee C. F., *Biotechnol. Prog.,* 9:366–373 (1999)], or proline isomerase [Schmid, *Current Biology,* 5:933–944 (1995)]. These and other such proteins are often capable of degrading the product quality or function of the desired recombinant protein(s) being expressed, for instance, by undesired cleavage, carbohydrate modification (glycoprotein modification)[Wittwer A., and Howard, S. C., *Biochem.,* 29:4175–4180 (1990); Hart, *Curr. Op. Cell Biol.,* 4:1017–1023 (1992); Goochee, et al., *Bio/Technology,* 9:1347–1355 (1991)], or protein structure modification (such as folding or aggregation). By decreasing or inhibiting apoptosis in the cell culture, the present methods can decrease the number or presence of such adverse proteases in the culture media and protect the expressed protein of interest against proteolytic degradation.

The methods herein can further be employed to increase transfection efficiency and viability of cells during transfection. Reagents used in various transfection techniques, such as Lipofectamine or DMRIE-C (Gibco), can be relatively toxic to the cells when used in higher concentrations. The use of higher concentrations of transfection reagents, however, would be particularly helpful to achieve higher transfection efficiencies. The expression of apoptosis inhibitor and/or the addition of apoptosis inhibitor directly to the cell culture medium can be used to reduce or inhibit cell death even when such higher concentrations of a transfection reagent are selected. The use of apoptosis inhibitor in this manner can result in higher transfection efficiency and higher yield of the recombinant protein of interest.

The methods disclosed can be further used to express proteins of interest which are proteins that, themselves, induce apoptosis. Such proteins like Apo-2 ligand/TRAIL or Fas ligand, can trigger apoptosis when expressed in cells. The presence of apoptosis inhibitor(s), in accordance with the present methods, may block such apoptotic activity and allow for improved expression of the protein of interest.

In addition, the methods can be used to increase the viability of cells undergoing freezing/storage/thawing procedures. During these procedures generally cells can lose viability. The presence of apoptosis inhibitor(s) expressed in cells (or added to the cell culture media) can provide for increased cell viability and aid in reducing or eliminating the variability in cell viabilities between aliquots or vials of cells.

The methods according to the present invention are described in further detail below.

The DNA encoding the protein of interest may be obtained from a variety of sources, for instance, from any cDNA library prepared from tissue believed to possess its mRNA and to express it at a detectable level. The gene encoding the protein of interest may also be obtained from a genomic library or by oligonucleotide synthesis. Screening such a cDNA or genomic library with a selected probe may be conducted using standard procedures, such as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding the protein of interest is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., *PCR Primer: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1995)].

Various proteins of interest have been specifically referred to above and their respective gene sequences are generally known and publicly available.

Genes encoding various apoptosis inhibitors have also been described in the literature [see e.g., Clem R. J. et al., *Science,* 254, 1388–1390 (1991); Duan, H. et al., *J. Biol. Chemistry,* 271, 16720–16724 (1996); Pan, G. et al., *J. Biol. Chemistry,* 273, 5841–5845 (1998); Vaux, D. L. et al., *Science,* 258, 1955–1957 (1998); Tsujimoto et al., *Proc. Natl. Acad. Sci.,* 83:5214–5218 (1986)]. The methods of the present invention contemplate the use of a single apoptosis inhibitor-encoding gene as well as the use of a combination of two or more apoptosis inhibitor-encoding genes. Potentially, the expression of two or more types of apoptosis inhibitor(s) in a host cell may be beneficial in controlling apoptosis in the cell culture. One skilled in the art can monitor the quantity or amount of apoptosis inhibitor being expressed by the host cells, such as by a Western blot analysis using an antibody that recognizes the apoptosis inhibitor. The quantity or amount of apoptosis inhibitor, as well as the timing of its expression, can be regulated or

monitored, for instance, by choosing a vector with an inducible promoter.

When selecting an apoptosis inhibitor for use in the claimed methods, those skilled in the art will appreciate that various apoptosis inhibitor molecules may act upon different intracellular components of the signaling pathway which leads to cell death. The pathways involved in cell death comprise a family of cysteine proteases, called caspases, that are related to the mammalian interleukin-1 beta converting enzyme (caspase-1) and to Ced-3, the product of a gene of *C. elegans*. It is believed that such caspase molecules can act at at least two different levels. Initiator caspases are typically "upstream" molecules that are activated in response to stimuli indicating that the cell has been stressed, damaged, or received some form of signal to initiate cell death by apoptosis. An example of such an upstream caspase is caspase-8. Initiator caspases can then, in turn, cleave and activate another family of "downstream" caspases, such as caspase-3. Depending upon the nature of the apoptotic stimulus as well as the cell type, only a portion of the signaling pathway may be involved in the signaling mechanism and execution of cell death. For example, certain apoptosis inhibitors, such as CrmA, are believed to act upon caspases, such as caspase-8, located upstream and are usually directly activated by death receptor binding to ligand. Other apoptosis inhibitors are believed to act upon other caspases located downstream in the intracellular signaling pathway. Thus, it is is presently believed that inhibitors of those molecule(s) that are effectively engaged (such as actively engaged in the signal transmission) in the cell death apparatus in a selected cell will be effective as apoptosis inhibitors, as described herein. Applicants do note, however, that those skilled in the art will understand that in such signaling pathways, there is point at which the cell is "committed" to cell death, and once the signaling pathway has transmitted a signal(s) to the point where the cell is committed to cell death, apoptosis inhibitor molecules, like those described herein, may not be effective in inhibiting or preventing the apoptosis of the "committed" cell.

The cytokine response modifier, CrmA, is a 38 kDa serpin identified from cowpox virus that has been reported to inhibit apoptosis in several systems [Gagliardini et al., *Science*, 263:826–828 (1994); Tewari et al., *J. Biol. Chem.*, 270:3255–3260 (1995)]. CrmA has been evaluated as an inhibitor of caspase-1 and caspase-8 [Nicholson et al., *Nature*, 376:37–43 (1995); Zhou et al., *J. Biol. Chem.*, 272:7797–7800 (1997)]. In some studies conducted by Applicants, it was observed that overexpression of CrmA in CHO dhfr+cells was unable to substantially delay cell death in the environment of a bioreactor. This result suggested that in this particular CHO cell system selected by Applicants, neither caspase-1 nor caspase-8 were actively involved in the cell death pathway of those particular cultured cells. Accordingly, to achieve the desired effects described herein, it is preferred to select an apoptosis inhibitor molecule which acts downstream in the selected host cell's cell death signaling pathway, but prior to the point where the cell has been committed to cell death.

The nucleic acids (e.g., cDNA or genomic DNA) encoding the protein of interest and the apoptosis inhibitor may be inserted into replicable vector(s) for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below. Optional signal sequences, origins of replication, marker genes, enhancer elements and transcription terminator sequences that may be employed are known in the art and described in further detail in WO97/25428.

Techniques for inserting such genes into vectors are well known to the skilled artisan and such techniques can be accomplished without undue experimentation. Construction of suitable vectors can employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required. Techniques known in the art may be employed. [See, e.g., Messing et al., *Nucleic Acids Res.*, 9:309 (1981); Maxam et al., *Methods in Enzymology*, 65:499 (1980)].

The gene encoding the apoptosis inhibitor and the gene encoding the protein of interest may be inserted into a single vector (co-transfected), or be inserted into two separate or different vectors. Preferably, the respective genes are inserted into two separate vectors. Each such vector will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, puromycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the encoding nucleic acid, such as DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980). A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid YRp7 [Stinchcomb et al., *Nature*, 282:39 (1979); Kingsman et al., *Gene*, 7:141 (1979); Tschemper et al., *Gene*, 10:157 (1980)]. The trp1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, *Genetics*, 85:12 (1977)].

In the methods employing a first vector comprising an apoptosis inhibitor gene and a second vector comprising a gene encoding the protein of interest, it is preferred that the first and second vector carry different selection markers. For example, a vector comprising the apoptosis inhibitor gene might carry a selection gene to confer ampicillin resistance while the vector comprising the gene encoding the protein of interest might carry a selection gene to confer methotrexate resistance.

Expression vectors usually also contain a promoter that is recognized by the host organism and is operably linked to the inserted nucleic acid sequence(s) described above. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to the encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector.

Promoters suitable for use with prokaryotic and eukaryotic hosts are known in the art, and are described in further detail in WO97/25428.

Expression vectors that provide for the transient expression of DNA encoding the protein of interest may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired protein encoded by the expression vector [Sambrook et al., supra]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of proteins encoded by cloned DNAs, as well as for the rapid screening of such proteins for desired biological or physiological properties.

Host cells are transfected or transformed with the above-described expression vectors for production of the protein of interest and cultured in nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell. As described above, the use of an apoptosis inhibitor gene (or adding an apoptosis inhibitor molecule directly to the culture media) may improve transfection efficiency. It is believed that use of such apoptosis inhibitor(s) will allow for use of increased amounts of transfection reagents, such as Lipofectamine or DMRIE-C (as described in the Examples below).

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with Agrobacterium tumefaciens is used for transformation of certain plant cells, as described by Shaw et al., *Gene,* 23:315 (1983) and WO 89/05859 published Jun. 29, 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published Jan. 10, 1991. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, *Virology,* 52:456–457 (1978) can be employed. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., *J. Bact.,* 130:946 (1977) and Hsiao et al., *Proc. Natl. Acad. Sci. (USA),* 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., *Methods in Enzymology,* 185:527–537 (1990) and Mansour et al., *Nature,* 336:348–352 (1988).

Suitable host cells for expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *E. coli.* Various *E. coli* strains are publicly available, such as *E. coli* K12 strain MM294 (ATCC 31,446); *E. coli* X1776 (ATCC 31,537); *E. coli* strain W3110 (ATCC 27,325) and K5 772 (ATCC 53,635); Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., *Salmonella typhimurium,* Serratia, e.g., *Serratia marcescans,* and Shigella, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published Apr. 12, 1989), Pseudomonas such as *P. aeruginosa,* and Streptomyces.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable. *Saccharomyces cerevisiae* is a commonly used lower eukaryotic host microorganism.

Suitable host cells may be derived from multicellular organisms. Examples of invertebrate cells include insect cells such as Drosophila S2 and Spodoptera Sf9, as well as plant cells. Examples of useful mammalian host cell lines include Chinese hamster ovary (CHO) and COS cells. More specific examples include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.,* 36:59 (1977)); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA,* 77:4216 (1980)); dp12.CHO (EP 307,247 published Mar. 15, 1989), mouse sertoli cells (TM4, Mather, *Biol. Reprod.,* 23:243–251 (1980)); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); and mouse mammary tumor (MMT 060562, ATCC CCL51). The selection of the appropriate host cell is deemed to be within the skill in the art.

The selection of a particular apoptosis inhibitor to employ with a particular host cell and protein of interest can be made without undue experimentation by one of ordinary skill in the art.

Prokaryotic cells used to produce the protein of interest may be cultured in suitable culture media as described generally in Sambrook et al., supra. Particular forms of culture media that may be employed for culturing CHO are described further in the Examples below. Mammalian host cells used to produce the protein of interest may be cultured in a variety of culture media. Suitable culture conditions for mammalian cells are well known in the art (J. Immunol. Methods (1983)56:221–234) or can be easily determined by the skilled artisan (see, for example, *Animal Cell Culture: A Practical ApDroach* 2nd Ed., Rickwood, D. and Hames, B. D., eds. Oxford University Press, New York (1992)), and vary according to the particular host cell selected.

Examples of commercially available culture media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). In addition, any of the media described in Ham and Wallace,(1979) Meth. Enz., 58:44; Barnes and Sato,(1980) Anal. Biochem., 102:255; U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 5,122,469 or 4,560,655; International Publication Nos. WO 90/03430; and WO 87/00195 may be used. Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously

used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The necessary growth factors for a particular cell are readily determined empirically without undue experimentation, as described for example in *Mammalian Cell Culture* (Mather, J. P. ed., Plenum Press, N.Y. (1984), and Barnes and Sato, (1980) Cell, 22:649.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the protein of interest in recombinant vertebrate cell culture are described in Gething et al., *Nature,* 293:620–625 (1981); Mantei et al., *Nature,* 281:40–46 (1979); EP 117,060; and EP 117,058. In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in *Mammalian Cell Biotechnology: A Practical Approach,* M. Butler, ed. (IRL Press, 1991).

The amount of apoptosis inhibitor added directly, or admixed, to the culture media will depend on various factors, for instance, the type of apoptosis inhibitor molecule being employed, the type of host cell, culture conditions, etc. Determining the desired concentration of apoptosis inhibitor to be added to the culture media is within the skill in the art and can be ascertained empirically without undue experimentation. Preferably, an effective amount or desired concentration of apoptosis inhibitor added directly to the culture media is such that the apoptosis inhibitor penetrates into the host cell. The skilled artisan will readily appreciate that different apoptosis inhibitors may have different abilities to penetrate into the host cell, and therefore, one should choose a concentration which allows for such penetration into the host cell. There will typically be an upper range of concentration of apoptosis inhibitor which may not be desirable as the concentration approaches a range which is adverse or toxic to the host cells. As described in the Examples below, Applicants have found that z-VAD-fmk can inhibit apoptosis when added to cell cultures at a concentration of about 100 micromolar. A variety of apoptosis inhibitor compounds such as z-VAD-fmk, z-DEVD-fmk, B-D-fmk, and z-YVAD-fmk are available from vendors, such as Pharmingen and Enzyme Systems, Livermore, Calif.

The apoptosis inhibitor may be added directly into the culture media. The apoptosis inhibitor may be added at any point during the culturing of the cells. Optionally, the apoptosis inhibitor is added to the culture media at the beginning (at the time of initiating, day 0) of the cell culturing process. Preferably, such an apoptosis inhibitor would be added to the culture media during the culturing of the cells but prior to the point when induction of apoptosis occurs; typically, induction of apoptosis can be observed in large scale cell cultures on about day 3 or day 4 of the culture, and therefore, the apoptosis inhibitor will preferably be added prior to day 3 or day 4. Optionally, a desired quantity of apoptosis inhibitor is added throughout, or for the duration of, the cell culture, for instance, on a daily basis for the entire fermentation. As an example, for a 5 day culture, the apoptosis inhibitor could be added at day 0, and every 24 hours thereafter until the culture is terminated.

In one embodiment of the invention, the selected host cell is a CHO cell, preferably, a dp12.CHO cell, and the selected culture medium contains a basal medium component such as a DMEM/HAM F-12 based formulation (for composition of DMEM and HAM F12 media and especially serum free media, see culture media formulations in American Type Culture Collection Catalogue of Cell Lines and Hybridomas, Sixth Edition, 1988, pages 346–349) (the formulation of medium as described in U.S. Pat. No. 5,122,469 are particularly appropriate) with modified concentrations of some

components such as amino acids, salts, sugar, and vitamins, and optionally containing glycine, hypoxanthine, and thymidine; recombinant human insulin, hydrolyzed peptone, such as Primatone HS or Primatone RL (Sheffield, England), or the equivalent; a cell protective agent, such as Pluronic F68 or the equivalent pluronic polyol; Gentamycin; and trace elements. Preferably, the selected cell culture media is serum free.

The proteins of interest may be produced by growing the host cells under a variety of cell culture conditions. For instance, cell culture procedures for the large or small scale production of proteins are potentially useful within the context of the present invention. Procedures including, but not limited to, a fluidized bed bioreactor, hollow fiber bioreactor, roller bottle culture, or stirred tank bioreactor system may be used, in the later two systems, with or without microcarriers, and operated alternatively in a batch, fed-batch, or continuous mode.

In a preferred embodiment, the cell culture of the present invention is performed in a stirred tank bioreactor system and a fed batch culture procedure is employed. In the preferred bioreactor system, the size of the bioreactors are sufficiently large to produce the desired amount of protein of interest, such as 1,000 Liter or 12,000 Liter sizes, but are not limited to such sizes as much smaller (i.e., 2 Liter, 400 Liter) or larger (i.e., 25,000 Liter, 50,000 Liter) bioreactor vessels may be appropriate. In the preferred fed batch culture, the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed batch culture can include, for example, a semi-continuous fed batch culture, wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium. Fed batch culture is distinguished from simple batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed batch culture can be further distinguished from perfusion culturing insofar as the supernate is not removed from the culturing vessel during the process but at the termination of the culture process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers etc. and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cultured cells may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, the present invention contemplates a single step or multiple step culture procedure. In a single step culture, the host cells are inoculated into a culture environment and the method steps of the instant invention are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture is envisioned. In the multi-stage culture, cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

According to a preferred aspect of the invention, fed batch or continuous cell culture conditions are devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In the growth phase, cells are grown under

conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (dO_2) and the like, are those used with the particular host and will be apparent to the ordinarily skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO_2) or a base (e.g., Na_2CO_3 or NaOH). A suitable temperature range for culturing mammalian cells such as CHO cells is between about 30 to 38° C. and preferably about 37° C. and a suitable dO_2 is between 5–90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above, the production phase or step may be continuous with the inoculation or growth phase or step.

According to the present invention, the cell culture environment during the production phase of the cell culture is controlled. According to the steps of the presently disclosed methods, the concentration of apoptosis inhibitor in the culture medium can be manipulated such that the desired content and quality of the protein of interest is achieved and maintained in the resulting cell culture fluid. In a preferred aspect, the production phase of the cell culture is preceded by a transition phase of the cell culture in which expression of or addition of apoptosis inhibitor(s) for the production phase of the cell culture are engaged. Concentrations of apoptosis inhibitor(s) are preferably monitored in connection with other process parameters such as the osmolality of the production phase since osmolality can affect the cell specific productivity.

In any of the above-described methods, it is contemplated that it may be desirable to include a desired amount of agent like butyrate or TSA in the cell culture medium. Various forms of butyrate and its salts are known in the art, such as butyric acid and sodium butyrate, and are publicly available from sources such as Sigma Chemical Co. Butyrate has been reported in the literature to enhance the productivity and protein expression of cell cultures [Arts et al., *Biochem J.*, 310:171–176 (1995); Gorman et al., *Nucleic Acids Res.*, 11:7631–7648 (1983); Krugh, *Mol. Cell. Biochem.*, 42:65–82 (1982); Lamotte et al., *Cytotechnology,* 29:55–64 (1999); Chotigeat et al., *Cytotechnology,* 15:217–221 (1994)]. Trichostatin A (TSA) is an inhibitor of histone deacetylase and may act similarly to butyrate in enhancing the productivity and protein expression in cell cultures [Medina et al.,*Cancer Research,* 57:3697–3707 (1997)]. Although butyrate has some positive effects on protein expression, it is also appreciated in the art that at certain concentrations, butyrate can induce apoptosis in the cultured cells and thereby decrease viability of the culture as well as viable cell density [Hague et al., *Int. J. Cancer,* 55:498–505 (1993); Calabresse et al., *Biochim. Biophys. Res. Comm.*, 195:31–38 (1993); Fillipovich et al., *Biochim. Biophys. Res. Comm.,* 198:257–265 (1994); Medina et al., *Cancer Research,* 57:3697–3707 (1997)]. In the methods of the present invention, a desired amount of butyrate or TSA may be added to the cell culture at the onset of the production phase and more preferably, may be added to the cell culture after a temperature shift has been implemented. Butyrate or TSA can be added in a desired amount determined empirically by those skilled in the art, but preferably, butyrate is added to the cell culture at a concentration of about 1 to about 25 mM, and more preferably, at a concentration of about 1 to about 6 mM.

Expression of the protein of interest may be measured in a sample directly, for example, by ELISA, conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, *Proc. Natl. Acad. Sci. USA,* 77:5201–5205 (1980)], dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}p. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal.

It is usually necessary to recover or purify the protein of interest from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. The protein of interest thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cationexchange resin such as DEAE; chromatography on protein A Sepharose columns, chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; and gel filtration using, for example, Sephadex G-75.

The recovered or purified protein of interest will typically be analyzed by one or more of the following methods: SDS-polyacrylamide gel electrophoresis, HPLC, mass spectrometry of a tryptic digest, glycoprotein analysis and activity assays.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, unless otherwise indicated, is the American Type Culture Collection, Manassas, Va.

Example 1

Apoptotic Cell Death in Bioreactors

Serum free adapted, CHO (dhfr+) cells were scaled up and seeded at 1 million cells/ml in 2 liter bioreactors (n=2).

The cell culture media was a serum-free DMEM/Ham F-12 based medium containing recombinant human insulin and trace elements. Cells were grown at 37° C. with agitation set at 275 rpm. pH was kept at 7.2 and was automatically adjusted throughout the experiment. Bioreactors were sparged with a mixture of oxygen and air. This is a model system that mimics the conditions during large-scale production of therapeutic proteins.

Samples were taken every day to measure the following parameters: cell viability, caspase activity (Clontech), DNA fragmentation and annexin/PI binding (Chemicon). FIG. **1A** shows cell viability as determined (by Trypan blue exclusion) over the course of 5 days. FIG. **1B** shows that loss of cell viability over the 5 day period in culture was the result of apoptosis.

Example 2

Effects of Caspase-9 Dominant Negative in CHO Cells

Expression construct: C-terminal FLAG-tagged caspase-9-dominant negative ("caspase-9-DN") cDNA (Duan, H. et al., *J. Biol. Chemistry,* 271, 16720–16724 (1996); Pan, G. et al., *J. Biol. Chemistry,* 273, 5841–5845 (1998)) was subcloned in a mpsv splice donor as further described: 2 ug of mpsv vector (Genentech, Inc.) was digested with 5 U of EcoRI and 5 U of BamHI (Boehringer Mannheim) and 2 ul of buffer A (Boehringer Mannheim) in a total volume of 20 ul for 1 hour at 37° C. 2 ug of caspase-9-DN/pcDNA3 construct was digested with 5 U of HindIII and 5 U of XbaI with 2 ul of buffer B (Boehringer Mannheim) in a total volume of 20 ul for 1 hour at 37° C. After incubation, 1 ul of 1 mM dNTPs (Clontech) and 0.2 U of Klenow polymerase (Boehringer Mannheim) was added to each reaction and the incubation was continued for an additional 15 minutes at 37° C.

Aliquots of each digest were analyzed by 1% agarose gel electrophoresis. 1.2 kbp caspase-9-DN cDNA and linearized 9.7 kbp mpsv vector were cut out of the gel and the DNA was purified using GeneClean (Bio101, Inc.) according to the manufacturer's instructions.

Ligation of caspase-9-DN and mpsv vector: 50 ng of vector and 42 ng of insert were ligated in 10 ul 2×ligation buffer and 1 ul T4 DNA ligase in 20 ul total volume at room temperature for 5 minutes (Boehringer Mannheim).

Transformation: MaxEfficiency DH5alfa competent cells (Gibco BRL) were transformed with 2 ul ligation mixture according to manufacturer's instructions. Transformed cells were then plated on carbenicillin containing LB plates. Colonies were randomly picked and analyzed by restriction digest to identify a colony containing the correct construct. Colony #30 was chosen for further work.

Transfection: E25 producing CHO DP12 cells [as designated throughout the present application, "E25" refers to the transfected CHO cells expressing a humanized monoclonal antibody against human IgE; see Presta et al., *J. Immunology,* 151:2623–2632 (1993)] were chosen for transfection with mpsv/caspase-9-DN and mpsv vector. Transfection was done using LipofectAMINE Plus Reagent (Gibco BRL) and was performed as follows:

E25 cells grown in suspension were plated on 60 mm tissue culture dishes (1 million cells/plate) 24 hours prior to transfection in a serum-containing medium. DNA for transfection was quantified spectrophotometrically. Two ug of DNA was mixed with 250 ul serum-free medium and 8 ul of Plus reagent and incubated for 15 minutes at room temperature. Twelve ul of reagent were mixed with 250 ul of serum-free medium and directly added to the mixture followed by incubation for15 minutes at room temperature. The medium on top of the cells was replaced with 5 ml of fresh serum-free medium and the transfection mixture was added to the dish. Three hours post-transfection, medium was replaced with a serum-containing medium. 24 hours post-transfection, each transfected dish was split into 5 dishes and a selection pressure was applied by the addition of 5 ug/ml puromycin. Transfected clones (resistant to puromycin) began to appear about two weeks after transfection. Several clones were chosen for analysis of caspase-9-DN expression by Western blotting.

Western blot analysis: Selected clones were picked and transferred into a 24 well plate. When confluent, cells from each well were rinsed with PBS and lysed for 3 minutes in 100 ul lysis buffer (3% NP 40 in PBS). The lysates were centrifuged for 3 minutes at 12,000×g. Supernatant was collected, mixed with an equal volume of reducing 2×SDS loading buffer (Novex) and boiled for 3 minutes. Samples were stored at −20° C. Aliquots of the lysates were subjected to a protein assay to determine the total protein concentration using Micro BCA Protein Assay Reagent Kit (Pierce).

Aliquots of lysates corresponding to **3** ug of total protein were loaded on a 10% SDS Tris-glycine gel (Novex) and ran for 1½ hours. Proteins were transferred to an Immobilon-P transfer Membrane according to manufacturer's directions.

The membrane was probed with rabbit anti-caspase-9 serum (Pharmingen) followed by HRP conjugated goat-anti-rabbit antiserum and developed using ECL Western Blotting detection Reagent (Amersham). Clones with high as well as low expression of caspase-9-DN (clones 2 and 14) were selected for further characterization. See FIG. **2**.

Induction of apoptosis with staurosporine: Clones 2 and 14 expressing low and high levels of caspase-9-DN (respectively) were adapted to growth in serum-free medium in spinners. Clones were seeded in spinners at 1 million cells/ml and an apoptosis inducing agent, staurosporine (Sigma), was added at 1 uM final concentration. Aliquots of culture were analyzed for apoptosis by several assays: annexin/PI (Chemicon) to measure the % of apoptotic cells and by caspase-3 activity (Clontech) according to manufacturer's instructions. See FIGS. **3** and **4**.

The effect of caspase-9-DN expression on viability in 2 liter bioreactors: Serum free adapted, caspase-9-DN expressing clones 2 and 14, a vector control and untransfected E25 cells were scaled up and seeded at 1 million cells/ml in 2 liter bioreactors (n=2). The cell culture media was a serum-free DMEM/Ham F-12 based medium containing recombinant human insulin and trace elements. Cells were grown at 37° C. with agitation set at 275 rpm. pH was kept at 7.2 and was automatically adjusted throughout the experiment. Bioreactors were sparged with a mixture of oxygen and air. This is a model system that mimics the conditions during large-scale production of therapeutic proteins.

Samples were taken every day to measure the following parameters: cell viability, cell density, apoptosis, caspase-3 activation, glucose consumption, osmolality, lactate production and E25 titers. See FIGS. **5–8**.

The results show stable expression of caspase-9-DN in CHO cells expressing E25. The stable expression resulted in a resistance of the cells to an apoptosis inducing agent, staurosporine. The resistance was proportional to the expression levels of caspase-9-DN. In the environment of a bioreactor, the high expessing clone 14 showed dramatically

prolonged viability and viable cell count compared with the lower expressing clone 2, which showed only a moderate prolongation of viability and viable cell count. Prolongation of viability is reflected in the delayed onset of caspase-3 activation in clone 14 compared with the controls. Unexpected results were obtained in the assay for the amount of E25 antibody secreted into the medium. Although caspase-9-DN clone 14 resulted in superior prolongation of viability in the bioreactor than clone 2, clone 14 produced less protein of interest (E25 antibody). The data suggested that high expression of apoptosis inhibitor may not concomitantly delay cell death and increase yield of the protein of interest.

However, in another 2 liter bioreactor assay, a cell culture was similarly run as described above with the exception of the following changes: (1) caspase-9-expressing clone 14 and E25 control cells were seeded at 1 million cells/ml; and (2) the medium was a serum free, concentrated medium (used to enhance nutrient supply in the medium) based on DMEM/Ham F-12 with insulin and trace elements. The cell cultures were grown for 1 day at 37° C. and then temperature shifted to 33° C. On the third day, the pH of the cultures was shifted from pH 7.15 to pH 7.0, and the cultures were fed with concentrated DMEM/Ham F-12, glucose and protein hydrolysate medium in order to supply enough nutrients to support optimal growth.

The results are shown in FIGS. **17** and **18**. As illustrated in the graphs, caspase-9-DN expression resulted in prolongation of viability and increase in viable cell densities, as well as higher titers of the protein of interest (E25 antibody) as compared to the control. Under the conditions of fed-batch culture where nutrients were not limiting, the data showed that prolongation of viability and increase in viable cell densities were accompanied by a marked increase in product titer.

Example 3

Effects of Caspase Inhibitor z-VAD-fmk on Apoptosis

CHO (dhfr+) cells grown in suspension were seeded at 1 million cells/ml in 60 mm tissue culture dishes. The cell culture media was a serum-free DMEM/Ham F-12 based medium containing recombinant human insulin and trace elements. Viability of the culture on day 0 was 96%. Two plates were analyzed each day for viability by Trypan Blue exclusion and by annexin/PI binding (Clontech) and for viable cell density. The experiment was carried out for 10 days. A chemical inhibitor of caspases, z-VAD-fmk (Enzyme Systems Products) was dissolved in DMSO to make a 100 mM (1000×) stock and 4 ul was added to a 60 mm dish containing 4 ml of culture. The inhibitor was added 48 hours after the start of the experiment (prior to the onset of apoptosis) and a new aliquot of the z-VAD-fmk inhibitor was added every 24 hours. Controls were cultures without any addition and cultures with the addition of DMSO only.

The results are shown in FIGS. **9**–**10**.

The chemical compound, z-VAD-fmk, is a caspase inhibitor and when added to the culture at 100 uM concentration, resulted in an inhibition of caspase-3 activity and prolongation of cell viability.

Example 4

Expression of Baculovirus p35 in CHO Cells

Expression construct: The baculovirus p35 cDNA (Beidler, D. et al., *J. Biol. Chemistry,* 270,16526–16528 (1995); Clem, R. J. et al., *Science,* 254, 1388–1390 (1991)) was subcloned from a pcDNA3 vector (Invitrogen) into a CPC splice donor vector as follows: 2 ug of CPC vector (Genentech, Inc.) was linearized by digestion in 25 ul containing 7 U of EcoRI and 7 U of XbaI in High buffer (Boehringer Mannheim) for 2 hours at 37° C. Baculovirus p35 cDNA was cut out of the pcDNA 3 vector (Invitrogen) with the same restriction enzymes. An aliquot of each reaction was analyzed by electrophoresis in 1% agarose gel containing ethidium bromide. Bands corresponding to the linearized CPC vector (9.7 kbp) and p35 cDNA (0.9 kbp) were cut out of the gel and isolated using GeneClean (Bio 101, Inc.) according to manufacturer's instructions.

Ligation: 50 ng of vector and 25 ng of p35 cDNA were mixed with 10 ul of T4 ligation buffer and 1 ul T4 DNA ligase (Rapid DNA Ligation Kit, Boehringer Mannheim) in 20 ul total reaction volume. The reaction was incubated for 5 minutes at room temperature.

Transformation: 100 ul of Max Efficiency DH5alfa Competent cells (Boehringer Mannheim) were mixed with 2 ul of ligation mixture and incubated on ice for 30 minutes. Cells were heat-shocked for 45 seconds at 42° C. followed by incubation on ice for 2 minutes. 0.9 ml of LB medium was added to the cells and incubated for 1 hour at 37° C. with agitation. 100 ul of transformed cells were plated on LB agar plate with carbenicillin. Four clones were randomly picked and were grown overnight in 4 ml of LB+carbenicillin. The plasmid was isolated from these colonies using QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. Isolated plasmids were subjected to an analytical digest to confirm the correct construct.

Expression of baculovirus p35 in CHO cells: CHO (dhfr+) cells grown in a DMEM/Ham F-12 media containing 2% fetal bovine serum (Gibco), recombinant human insulin and trace elements were plated 48 hours prior to transfection at 2 million cells/100 m tissue culture dish. LipofectAMINE Plus Reagent (Gibco BRL) was used for transfection and was performed according to manufacturer's instructions. CHO cells were transfected with a p35 /CPC construct and CPC vector alone as a control. One transfected plate of each type was harvested 24 hours after transfection to assay the level of p35 expression in transient transfectants (Western blotting using anti-p35 rabbit polyclonal serum at 1:1000 dilution). Other tranfected plates were grown further and selection pressure (5 ug/ml puromycin) was applied 48 hours post-transfection. About two weeks later colonies resistant to puromycin developed and were adapted to serum free growth and scaled up for further analysis.

The effect of p35 expression on viability in 2 liter bioreactors: The serum free adapted clone expressing p35 and a vector control were scaled up and seeded at 1 million cells/ml in 2 liter bioreactors (n=2). The cell culture media was a serum-free DMEM/Ham F-12 based medium containing recombinant human insulin and trace elements. Cells were grown at 37° C. with agitation set at 275 rpm. pH was kept at 7.2 and was automatically adjusted throughout the experiment. Bioreactors were sparged with a mixture of oxygen and air. This is a model system that mimics the conditions during large-scale production of therapeutic proteins. Samples were taken every day to measure the following parameters: cell viability, cell density, apoptosis, caspase-3 activation, glucose consumption, osmolality and lactate production.

The results are shown in FIGS. **11**–**12**.

The results indicate that the apoptosis inhibitor, baculovirus p35, when expressed in CHO cells results in prolongation of viability in the environment of the bioreactor.

Example 5

Increased Transfection Efficiency and Expression of E25 Antibody in Transient Transfections Using Caspase-9-DN

Serum free adapted CHO DP12 cells were seeded at 1.5 million cells/ml in untreated 12 well tissue culture plates in medium based on DMEM/HAM F-12 with modified concentrations of some components and containing recombinant human insulin, trace elements and serum. Transfection was performed using DMRIE-C (Gibco BRL) according to manufacturer's instructions. Caspase-9-DN expressing clone 14 was transfected next to controls which were CHO DP12 cells and E25 cells (CHO DP12, expressing E25).

Red shifted GFP expressing vector (Quantum Biotechnologies Inc.) was co-transfected with a DNase expressing vector [Shak, S. et al., (1990), *Proc Natl. Acad. Sci USA*, 87:9188–9192)]. 24 hours post-transfection, propidium iodide was added to an aliquot of the culture and total and viable transfection efficiencies were assayed by flow cytometry on FACSCalibur (Becton Dickinson). Five days after transfection, a sample of the medium was subjected for DNase titer analysis using ELISA.

The data indicated (FIG. **13**) that transfection reagent, in our experiment DMRIE-C, can be toxic to cells when used at higher concentrations (above 6 ul). In FIG. **14**, caspase-9-DN clone 14 shows (in all concentrations of DMRIE-C tested) higher total and viable transfection efficiencies than controls. The transfection efficiency of clone 14 increased with the amount of transfection reagent and reached maximum at 12 ul of DMRIE-C, at which concentration both controls already started to show a decrease in transfection efficiency. It is possible that transfection efficiency of clone 14 will increase even further when higher than currently tested amount of DMRIE-C is used. The increase in transfection efficiency of clone 14 was reflected in the specific productivity (DNase titer/total LDH) of the culture and in DNase titer (FIGS. **15**, **16**), both of which were increased up to four-fold compared with the controls.

Example 6

Effect of Caspase 9-DN Expression on Viability and Viable Cell Number After Thawing a Frozen Culture

2×10^7 cells of caspase-9-DN expressing clone 14 and E25 control cells were frozen in a freezing medium (1 g/L methylcellulose in modified DMEM/Ham F-12 and 10% DMSO) and stored at −80° C. for an extended period of time. On the day of the experiment, vials of frozen cells were taken out of the freezer, thawed at 37° C. and added to a spinner with a pre-warmed growth medium (modified DMEM/Ham F-12). Cells were cultured for 8 days and assayed for viability and viable cell density.

The results are shown in FIGS. **19** and **20**. The results indicate that caspase-9-DN expressing cells maintained higher viability and viable cell count than the control E25 cells. Thus, expression of caspase-9-DN in the CHO cells had a beneficial effect on viability and viable cell densities upon thawing the frozen cell cultures.

Example 7

Caspase-9-DN Expressing Cells Show Resistance to Butyrate

The following study was conducted to examine whether caspase-9-DN expression affects resistance of the cells to potential adverse effects of butyrate.

Caspase-9-DN expressing clone 14 and E25 control cells were seeded at 1×10^6 cells/ml in 60 mm tissue culture dishes. Each dish contained 4 ml of culture medium. Cultures were grown at 37° C. in concentrated medium based on DMEM/Ham F-12 with insulin and trace elements. Cultures were temperature shifted to 33° C. on the second day and butyrate was added on the third day at varying final concentrations (0, 1, 2, 3, 5, 10 mM) (n=2). Viability of the cultures and titers were assayed daily.

The results are shown in FIGS. **21** and **22**. The results showed that E25 control cells lose viability more rapidly than caspase-9-DN expressing cells (see FIG. **21**, day 7 and day 9). This is reflected in the titers of protein of interest. Titers shown in FIG. **22** indicate that caspase-9-DN cells gave higher titers than 0 butyrate addition in cultures with 1 ,2 ,3 and 5 mM butyrate. On the other hand, titers of E25 controls improved with only 1 and 2 mM butyrate. The results suggest that caspase-9-DN expression protects cells from adverse effects of butyrate and can result in extended viability and higher titers.

What is claimed is:

1. A method of making recombinant proteins using one or more apoptosis inhibitors, comprising the steps of:

(a) providing a vector comprising a gene encoding caspase-9 dominant negative protein,

(b) providing a vector comprising a gene encoding a protein of interest,

(c) providing a Chinese hamster ovary (CHO) host cell,

(d) transforming or transfecting the host cell with the vector of steps (a) and (b),

(e) providing cell culture media,

(f) culturing the transformed or transfected host cell in the cell culture media under conditions sufficient for expression of the protein of interest and the caspase-9 dominant negative protein, and optionally

(g) recovering or purifying the protein of interest from the host cell and/or the cell culture media.

2. The method of claim **1** further comprising the step of admixing an additional apoptosis inhibitor into the cell culture media in steps (e) or (f).

3. The method of claim **1** wherein the vector of step (a) and the vector of step (b) are the same vector.

4. The method of claim **1** wherein the vectors of steps (a) and (b) are two separate vectors.

5. The method of claim **4** wherein the vectors of steps (a) and (b) comprise different antibiotic resistance selection markers.

6. The method of claim **1** wherein the host cells are cultured under conditions for transient expression of the protein of interest.

7. The method of claim **1** wherein the protein of interest comprises a protein which is capable of inducing apoptosis in a mammalian or non-mammalian cell.

8. The method of claim **1** wherein said cell culture media is serum-free media.

9. The method of claim **1** wherein said cell culture media comprises butyrate.

10. The method of claim **1** wherein after step (f), the host cell(s) and/or cell culture media is frozen and subsequently thawed.

11. A method of making recombinant proteins using one or more apoptosis inhibitors, comprising the steps of:

(a) providing a vector comprising a gene encoding a protein of interest,

(b) providing a Chinese hamster ovary (CHO) host cell comprising a gene encoding caspase-9 dominant negative protein,

(c) transforming or transfecting the host cell with the vector of step (a),

(d) providing cell culture media,

(e) culturing the transformed or transfected host cell in the cell culture media under conditions sufficient for expression of the protein of interest and the caspase-9 dominant negative protein and optionally

(f) recovering or purifying the protein of interest from the host cell and/or cell culture media.

12. The method of claim **11** wherein the gene encoding the caspase-9 dominant negative protein is stably integrated into the genome of the host cell.

13. The method of claim **11** further comprising the step of admixing an additional apoptosis inhibitor molecule into the cell culture media in steps (d) or (e).

14. The method of claim **11** wherein said cell culture media comprises butyrate.

15. The method of claim **11** wherein after step (e), the host cell(s) and/or cell culture media is frozen and subsequently thawed.

16. A method of making recombinant proteins using one or more apoptosis inhibitors, comprising the steps of:

(a) providing a vector comprising a gene encoding a protein of interest,

(b) providing a Chinese hamster ovary (CHO) host cell,

(c) transforming or transfecting the host cell with the vector of step (a),

(d) providing cell culture media,

(e) providing an amount of caspase inhibitor z-VAD-fmk,

(f) admixing the caspase inhibitor into the cell culture media,

(g) culturing the host cell in the cell culture media under conditions sufficient for expression of the protein of interest, and optionally

(h) recovering or purifying the protein of interest from the host cell and/or the cell culture media.

17. The method of claim **16** wherein after step (g), the host cell(s) and/or cell culture media is frozen and subsequently thawed.

18. A method of increasing yield of a protein of interest in a cell culture, comprising the steps of:

(a) providing a vector comprising a gene encoding caspase-9 dominant negative protein,

(b) providing a vector comprising a gene encoding a protein of interest,

(c) providing a Chinese hamster ovary (CHO) host cell,

(d) transforming or transfecting the host cell with the vector of steps (a) and (b),

(e) providing cell culture media,

(f) culturing the transformed or transfected host cell in the cell culture media under conditions sufficient for expression of the protein of interest and an amount of the caspase-9 dominant negative protein which is effective in increasing yield of the protein of interest, and optionally

(g) recovering or purifying the protein of interest from the host cell and/or the cell culture media.

19. The method of claim **18** wherein said cell culture media is serum-free media.

20. The method of claim **18** wherein after step (f), the host cell(s) and/or cell culture media is frozen and subsequently thawed.

21. A method of prolonging host cell viability in a cell culture, comprising the steps of:

(a) providing a vector comprising a gene encoding caspase-9 dominant negative protein,

(b) providing a vector comprising a gene encoding a protein of interest,

(c)providing a Chinese hamster ovary (CHO) host cell,

(d) transforming or transfecting the host cell with the vector of steps (a) and (b),

(e) providing cell culture media,

(f) culturing the transformed or transfected host cell in the cell culture media under conditions sufficient for expression of the protein of interest and an amount of caspase-9 dominant negative protein which is effective for prolonging viability of the host cells in the cell culture, and optionally

(g) recovering or purifying the protein of interest from the host cell and/or the cell culture media.

* * * * *

EXHIBIT X

US006610516B1

(12) **United States Patent**
Andersen et al.

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(45) **Date of Patent: *Aug. 26, 2003**

(54) **CELL CULTURE PROCESS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

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(58) **Field of Search** 530/395; 435/69.1, 435/69.6, 252.3, 358, 359, 70.1

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,560,655 A	12/1985	Baker
4,657,866 A	4/1987	Kumar
4,752,603 A	6/1988	Collen et al.
4,766,075 A	8/1988	Goeddel et al.
4,767,704 A	8/1988	Cleveland et al.
4,927,762 A	5/1990	Darfler
5,122,469 A	6/1992	Mather et al.
5,705,364 A	1/1998	Etcheverry et al.
5,721,121 A	2/1998	Etcheverry et al.

OTHER PUBLICATIONS

Parekh et al. Cell–Type–Specific and Site–Specific N–Glycosylation of Type I and Type II Human Tissue Plasminogen Activator, Biochemistry, 1989, vol. 28, No. 19, pp 7644–7662.*

U.S. patent application Ser. No. 09/470,849, Etcheverry et al., filed Jun. 6, 1995.

U.S. patent application Ser. No. 09/705,285, Etcheverry et al., filed Jan. 11, 2000.

U.S. patent application Ser. No. 09/723,625, Andersen et al., Nov. 27, 2000.

Mather, Jennie P. *Mammalian Cell Culture, the Use of Serum–Free Hormone–Supplemented Media*, Plenum Press, New York pps. vii–x, 1–15, 17–52, 129–150 (1984).

Rudinger, J., "Characteristics of the Amino Acids as Components of a Peptide Hormone Sequence" *Peptide Hormones*, J.A. Parsons, Baltimore:University Park Press pps. 6 (1976).

Wilhelm, J., et al., "Alterations in the Domain Structure of Tissue–Type Plasminogen Activator Change the Nature of Asparagine Glycosylation" *Bio/Technology* 8:321–324 (Apr. 1990).

Castro et al., "The macroheterogeneity of recombinant human interferon–gamma produced by Chinese–hamster ovary cells is affected by the protein and lipid content of the culture medium" *Biotechnology and Applied Biochemistry* (abstract only) 21(1):87–100 (1995).

Castro, P. et al., "The macroheterogeneity of recombinant human interferon–gamme produced by the Chinese–hamster ovary cells is affected by the protein and lipid content of the culture medium" *Biotechnology and Applied Biochemistry* 21(1):87–100 (1995).

Gebert and Gray, "Expression of FSH in CHO Cells. II. Stimulation of hFSH expression levels by defined medium supplements." *Cytotechnology* (abtract only) 17:13–19 (1995).

Gebert, C.A. and Gray, P.P., "Expression of FSH in CHO cells. II. Stimulation of hFSH expression levels by defined medium supplements" *Cytotechnology* 17:13–19 (1995).

Hosoi et al., "Modulation of oligosaccharide structure of a pro–urokinase derivative (pro–UKΔGS1) by changing culture conditions of a lymphoblastoid cell line Namalwa KJM–1 adapted to serum–free medium" *Cytotechnology* 19:125–135 (1996).

Kimura and Miller, "Glycosylation of CHO–derived recombinant tPA produced under elevated pCO–c" *Biotechnology Progress* (abstract only) 13(3):311–317 (1997).

Kimura, R. and Miller, W., "Glycosylation of CHO–derived recombinant tPA produced under elevated pCO–2" *Biotechnology Progess* 13(3):311–317 (1997).

Lamotte et al., "Na–butyrate increases the production and alpha2, 6–sialylation of recombinant interferon–gamma expressed by alpha2, 6–sialyltransferase engineered CHO cells" *Cytotechnology* (abstract only) 29:55–64 (1999).

(List continued on next page.)

Primary Examiner—Karen Cochrane Carlson
Assistant Examiner—Rita Mitra
(74) *Attorney, Agent, or Firm*—Janet E. Hasak

(57) **ABSTRACT**

A glycoprotein is produced by a process comprising culturing mammalian host cells expressing nucleic acid encoding a glycoprotein in the presence of (a) a factor that modifies growth state in a cell culture, (b) a divalent metal cation that can adopt and prefers an octahedral coordination geometry, and/or (c) a plasma component. In this process, the occupancy of an N-linked glycosylation site occupied only in a fraction of a glycoprotein is enhanced. Such culturing is preferably carried out at a temperature of between about 30° C. and 35° C. and/or in the presence of up to about 2 mM of a butyrate salt and/or in the presence of a cell-cycle inhibitor.

1 Claim, 8 Drawing Sheets

OTHER PUBLICATIONS

Lamotte, D. et al., "Na–butyrate increases the production and alpha2, 6–sialylation of recombinant interferon–gamma expressed by alpha2, 6–sialyltransferase engineered CHO cells" *Cytotechnology* 29:55–64 (1999).

Madoiwa, S. et al., "Effect of carbohydrate side chain of tissue–type plasminogen activator on its interaction with plasminogen activator inhibitor–1" *Fibrinolysis & Proteolysis* 12(1):17–22 (1998).

Nabi and Dennis, "The extent of polylactosamine glycosylation of MDCK LAMP–2 is determined by its Golgi residence time" *Glycobiology* 8(9):947–953 (1998).

West and Brownstein, "EDTA treatment alters protein glycosylation in the cellular slime mold dictyostelium–discoideum" *Experimental Cell Research* (abstract only) 175:26–36 (1988).

West, C.M. and Brownstein, S.A., "EDTA Treatment alters protein glycosylation in the cellular slime mold dictyostelium–discoideum" *Experimental Cell Research* 175:26–36 (1988).

Woodfork, K. et al., "Inhibition of ATP–sensitive potassium channels causes reversible cell–cycle arrest of human breast cancer cells in tissue culture" *Journal of Cellular Physiology* 162(2):163–171 (1995).

* cited by examiner

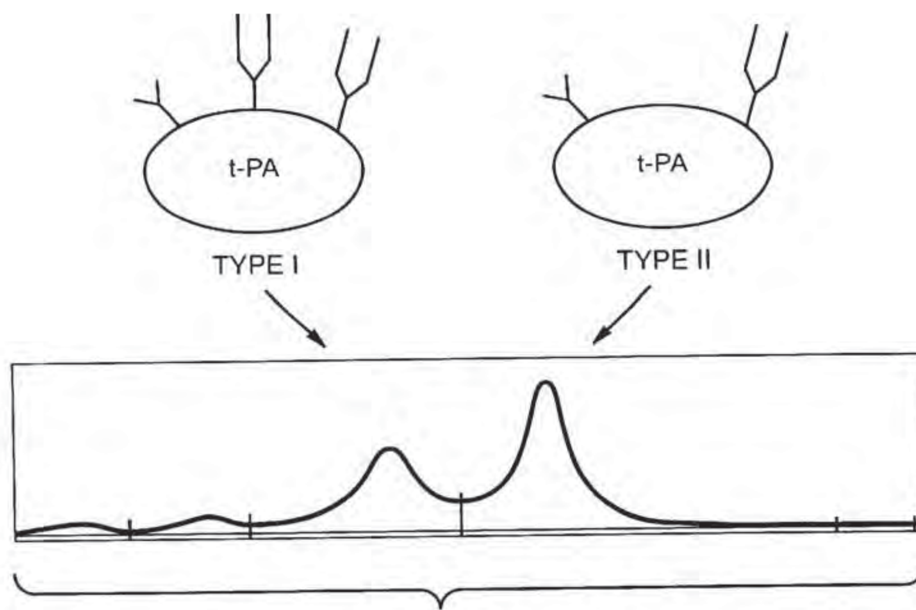

FIG._1

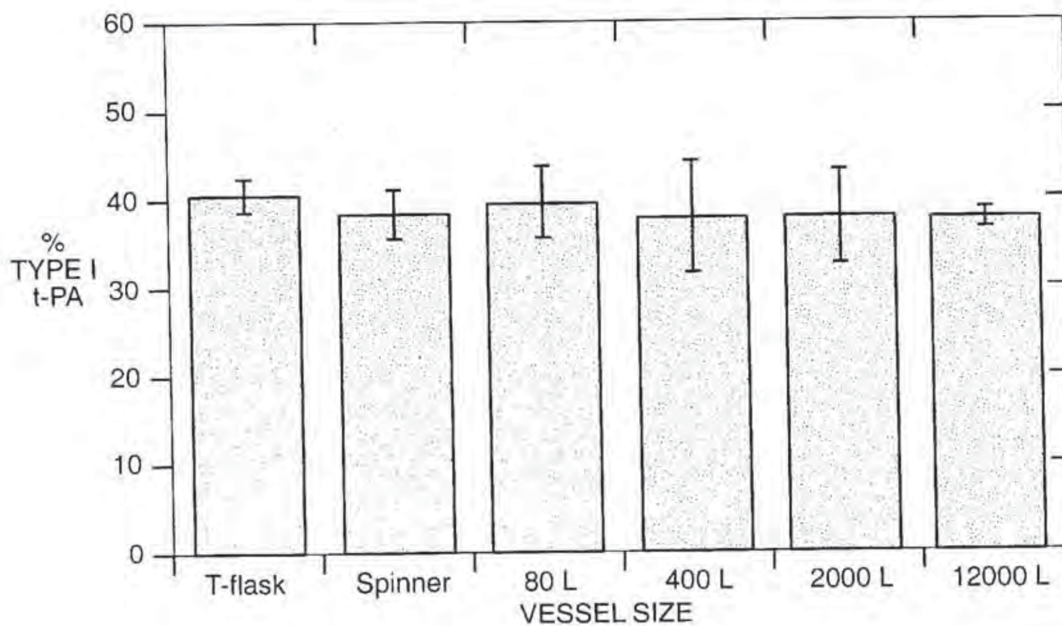

FIG._2

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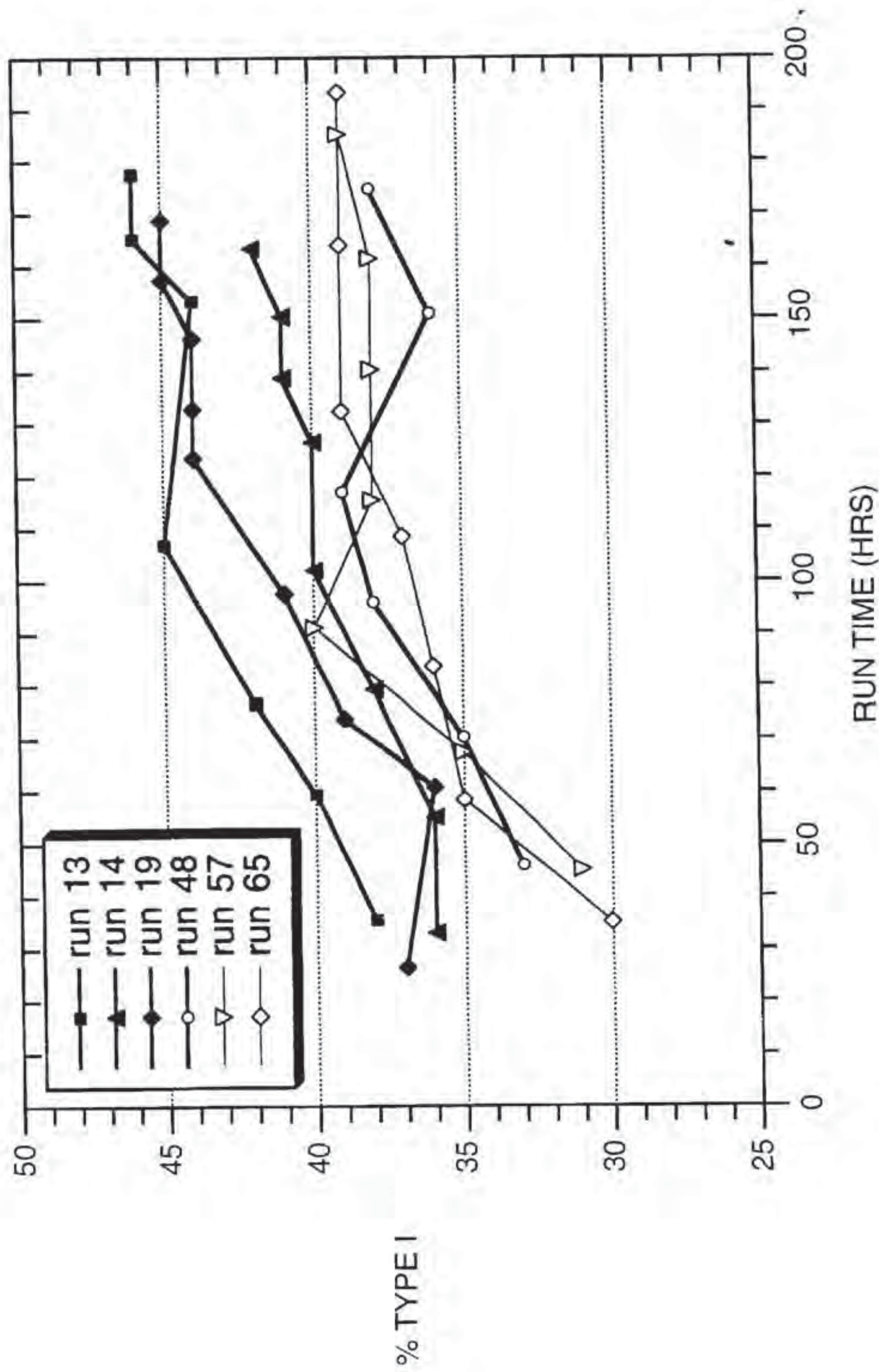

FIG._3

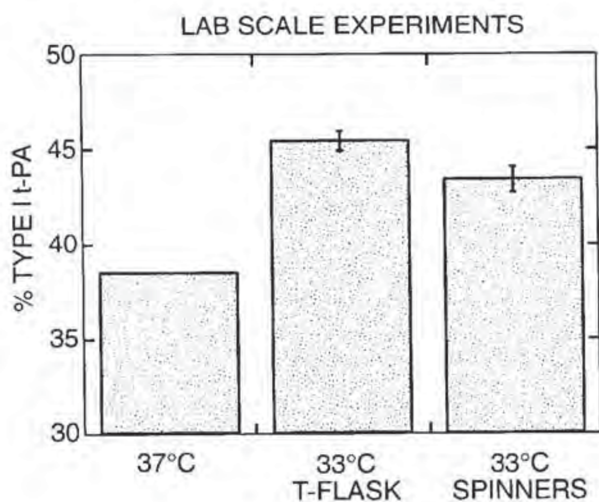

FIG._4A

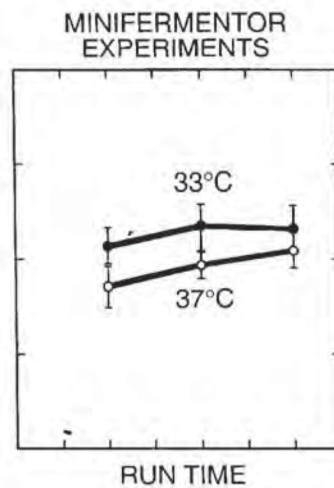

FIG._4B

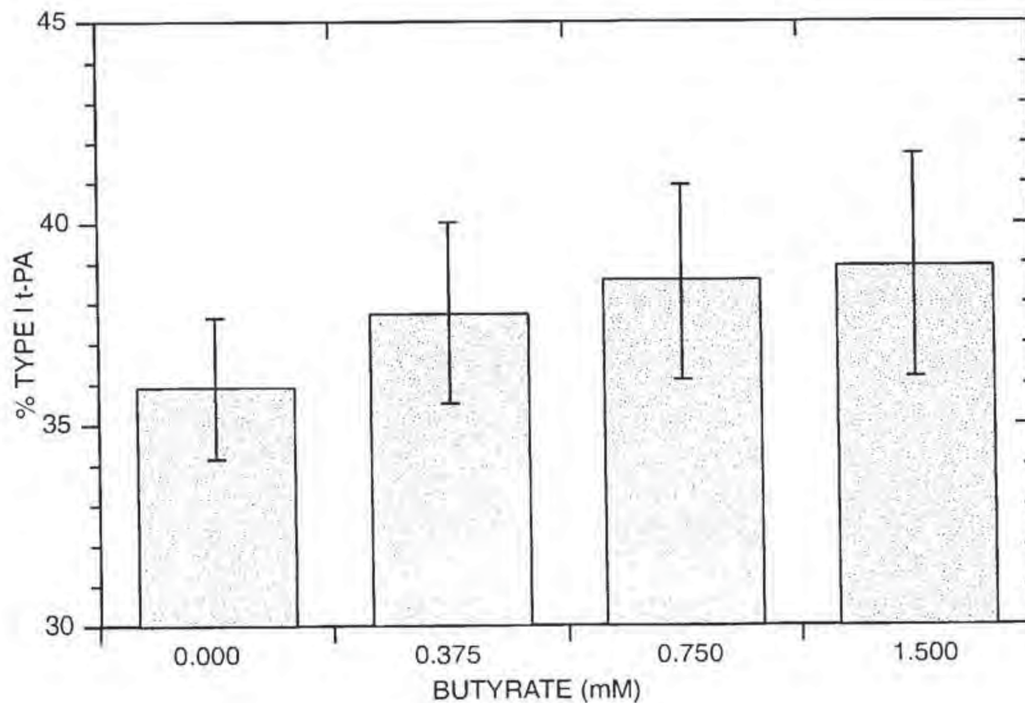

FIG._5

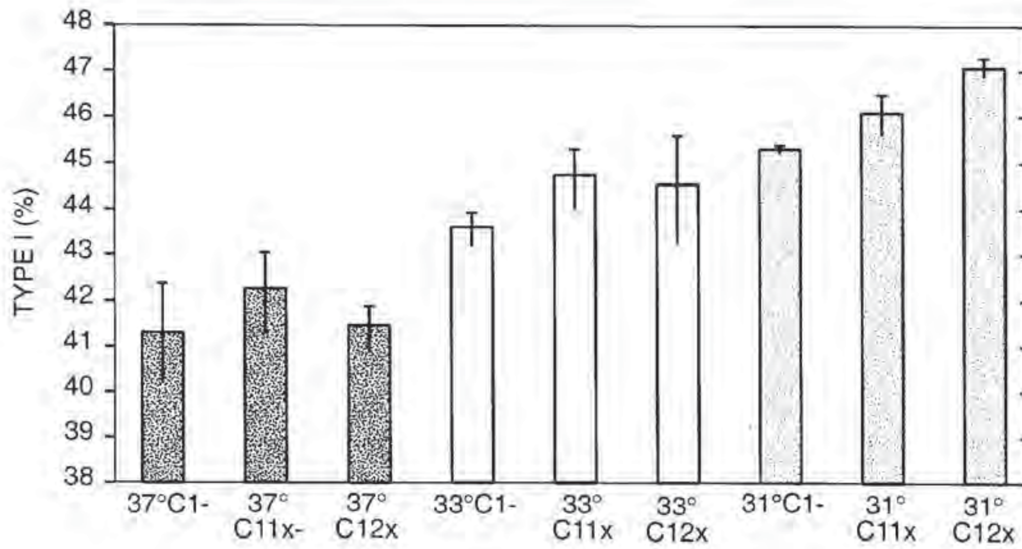

FIG._6

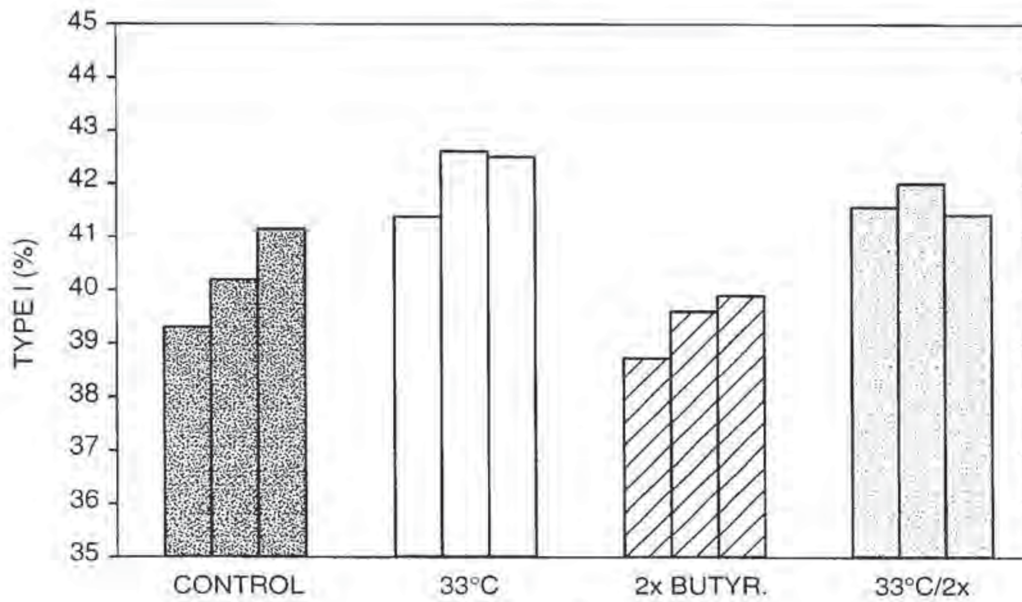

FIG._7

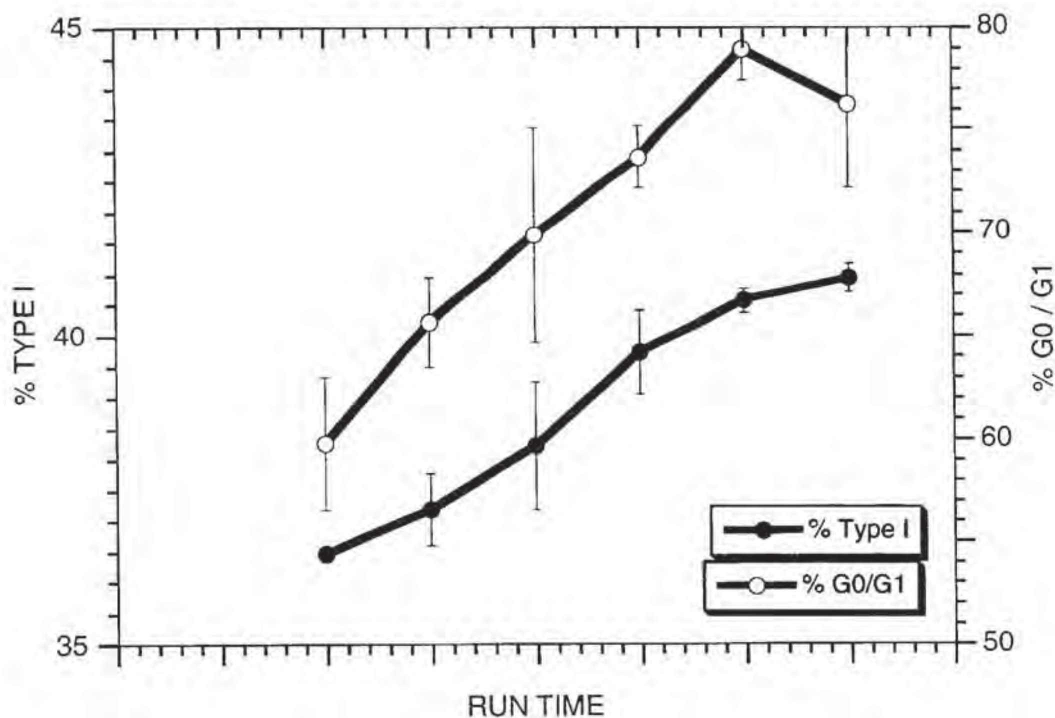

FIG._8

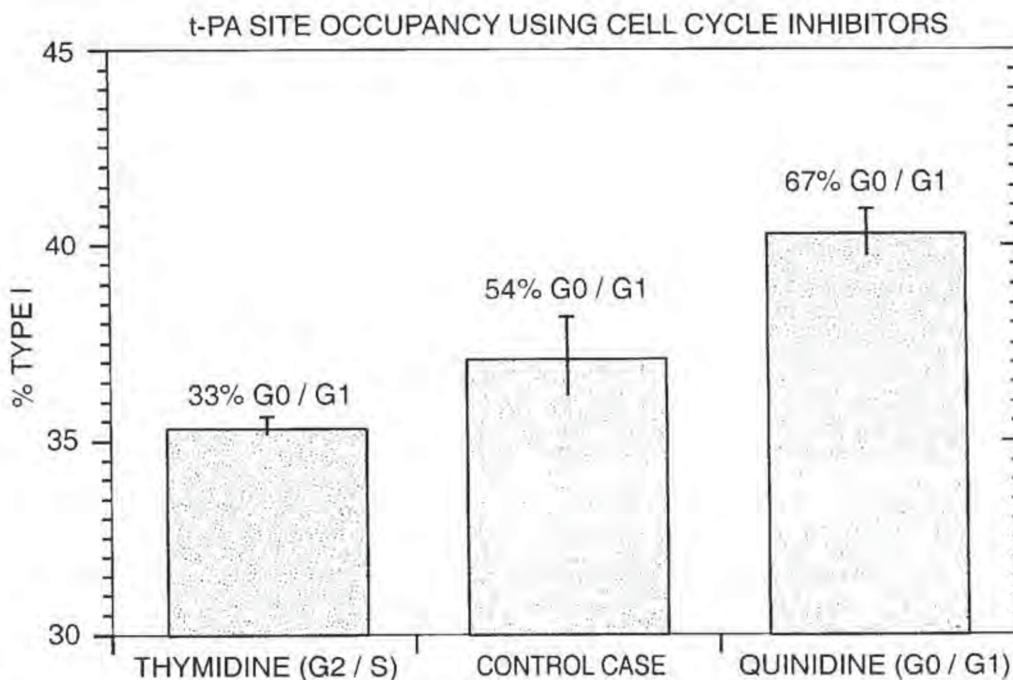

FIG._9

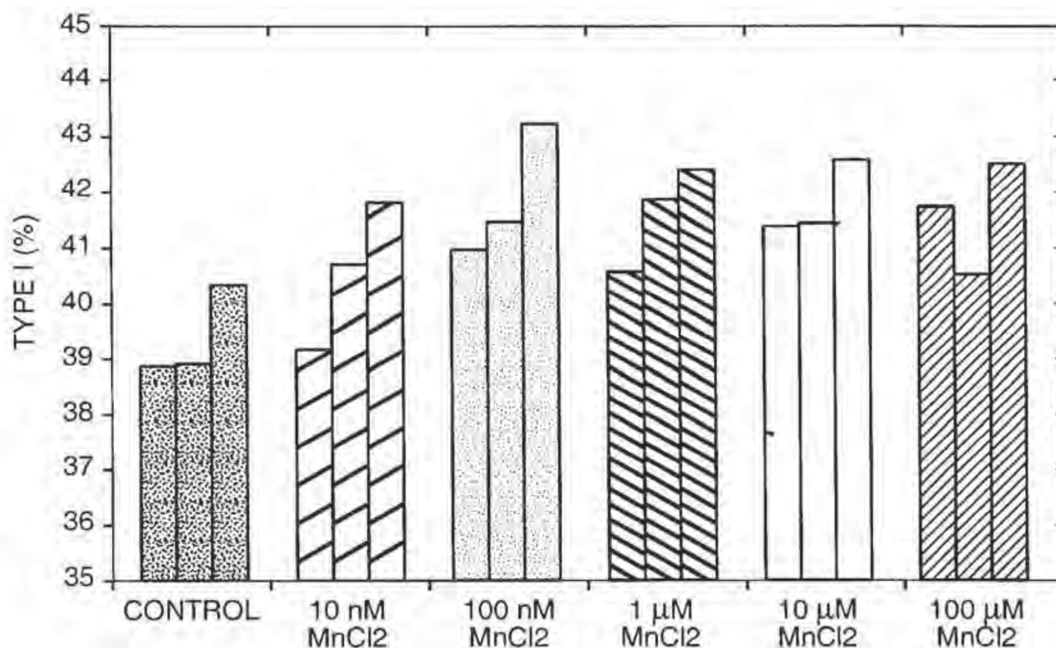

FIG._10A

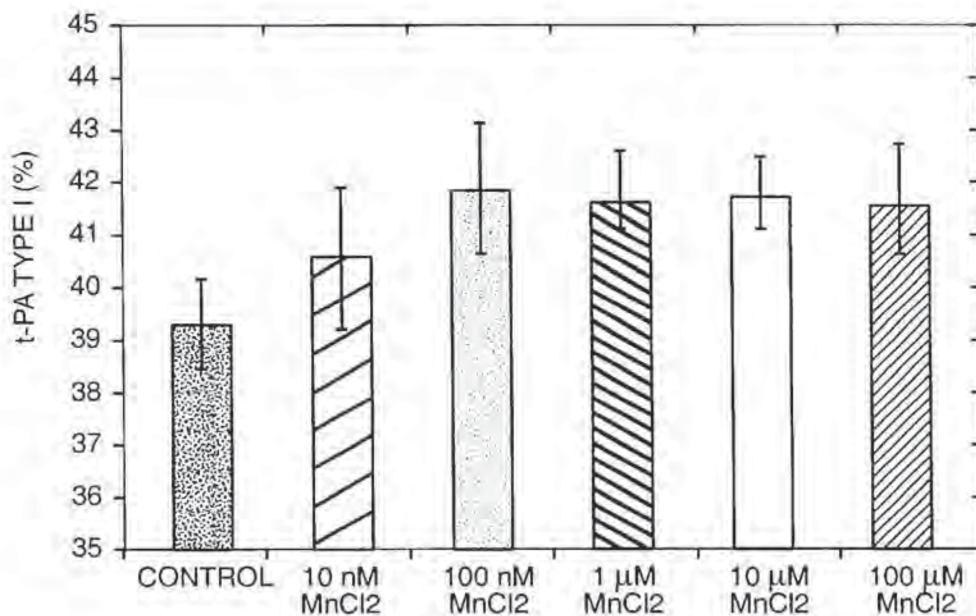

FIG._10B

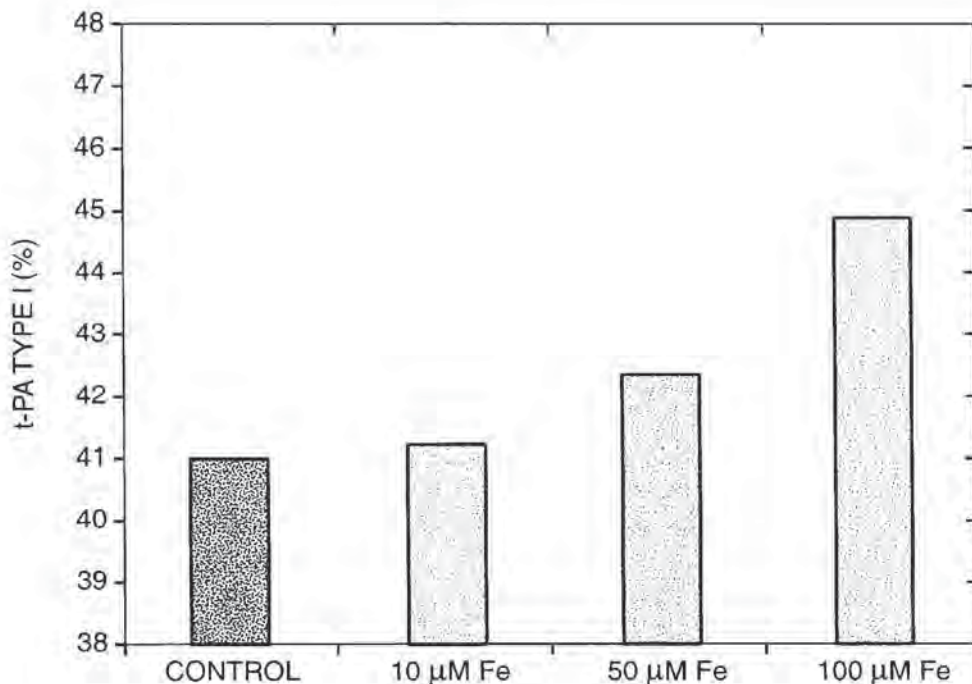

FIG._11

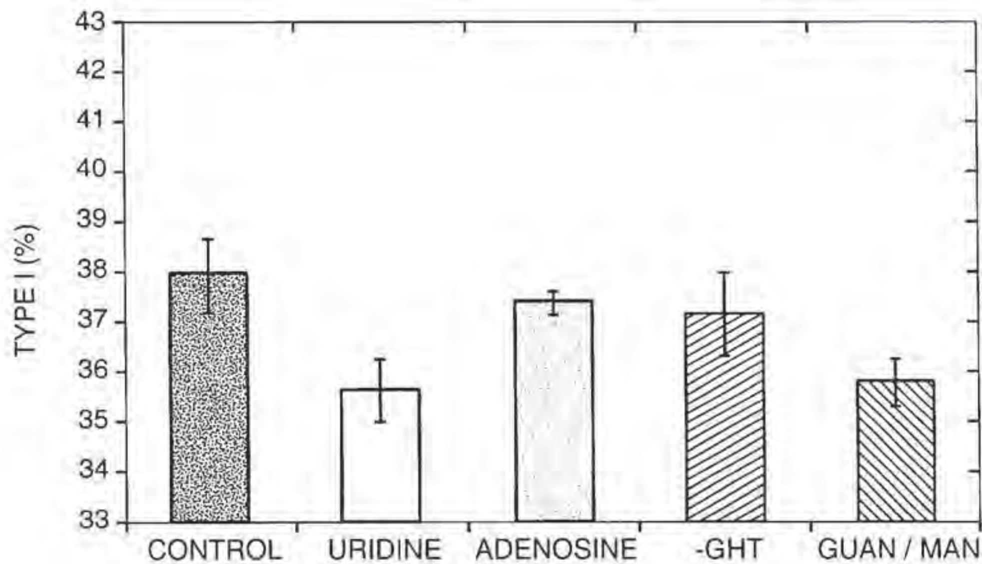

FIG._12

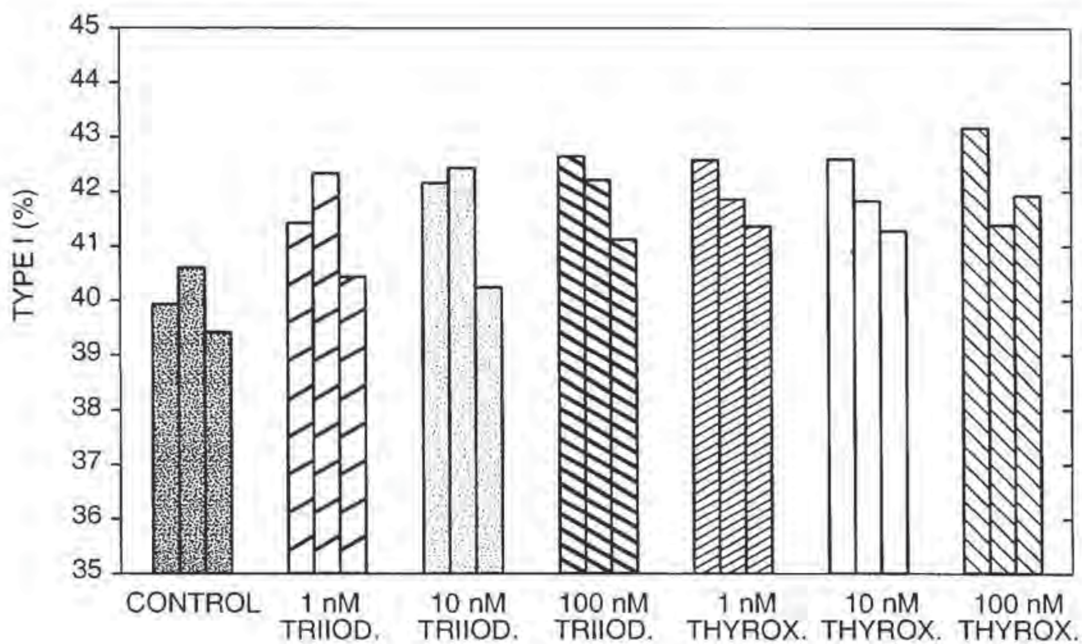

FIG._13A

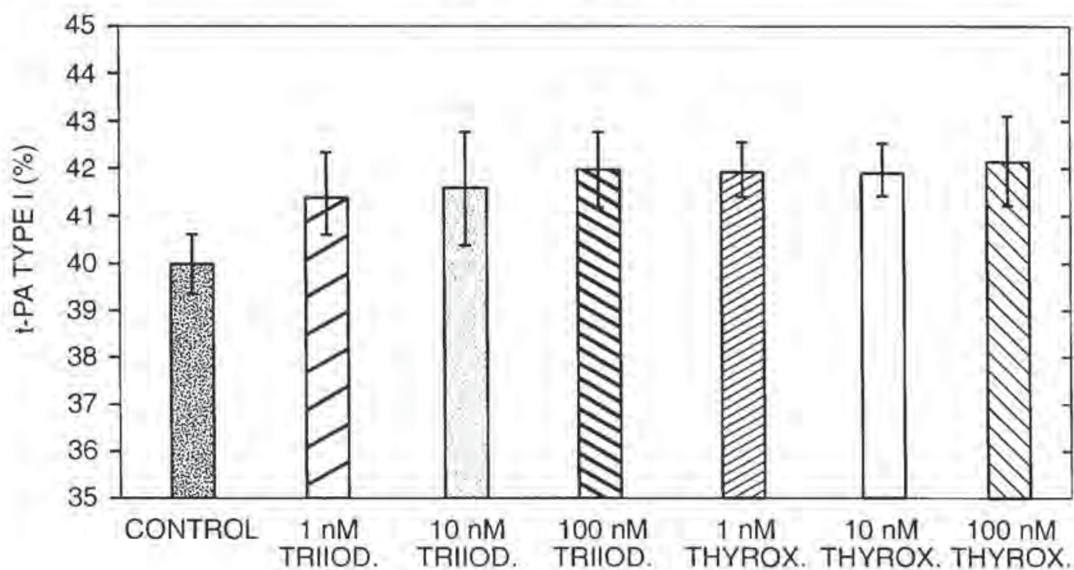

FIG._13B

CELL CULTURE PROCESS

This is a divisional application claiming priority to application Ser. No. 09/553,924, filed Apr. 21, 2000, which claims priority to U.S. Provisional Application Serial No. 60/131,076, filed Apr. 26, 1999, the entire disclosure of which is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention concerns a process for the production of glycoproteins in mammalian cell culture. More specifically, the invention provides a process for producing glycoproteins in mammalian cells that results in enhanced occupancy of an N-linked glycosylation site occupied only in a fraction of a glycoprotein. A process for increasing the fraction of Type I tissue plasminogen activator (t-PA) in a mammalian cell culture is specifically disclosed.

2. Description of Related Disclosures and Technology Glycoproteins

Glycoproteins, many of which have been produced by techniques of recombinant DNA technology, are of great importance as diagnostic and therapeutic agents. In a eukaryotic cell environment, glycosylation is attached to a secreted or membrane-spanning protein by co- and post-translational modification. Proteins destined for the cell surface are first co-translationally translocated into the lumen of the endoplasmic reticulum (ER) mediated by a signal sequence at or near the amino terminus of the nascent chain. Inside the ER, the signal sequence is usually removed and a high-mannose core oligosaccharide unit is attached to the asparagine (N) residue(s) present as part of the sequence Asn-X-Ser/Thr, where X is any amino acid except, perhaps, proline.

The efficiency of this co-translational glycosylation step is dependent on the presentation of an appropriate conformation of the peptide chain as it enters the endoplasmic reticulum (Imperiali and O'Connor, *Pure & Applied Chem.*, 70: 33–40 (1998)). Potential N-linked glycosylation sites may no longer be accessible after the protein has folded (Kornfeld & Kornfeld, *Ann Rev. Biochem.* 54:631–664 (1985)). Proteins next move from the ER to the Golgi apparatus where further modifications, such as sulfation and processing of the high-mannose oligosaccharide chain to a complex-type oligosaccharide, occur and the proteins are directed to their proper destinations.

N-linked oligosaccharides can have a profound impact on the pharmaceutical properties of glycoprotein therapeutics (e.g., in vivo half-life and bioactivity). Different bioprocess parameters (e.g., bioreactor type, pH, media composition, and ammonia) have been shown to affect protein glycosylation significantly. Changes in terminal glycosylation (sialylation and galactosylation) and N-glycan branching are the most frequently observed alterations.

The Carbohydrate Structure of Tissue Plasminogen Activator

Tissue plasminogen activator (t-PA), a glycoprotein, is a multidomain serine protease whose physiological role is to convert plasminogen to plasmin, and thus to initiate or accelerate the process of fibrinolysis. Initial clinical interest in t-PA was raised because of its relatively high activity in the presence, as compared to the absence, of fibrin. Wild-type t-PA is a poor enzyme in the absence of fibrin, but the presence of fibrin strikingly enhances its ability to activate plasminogen. Recombinant human t-PA is used therapeutically as a fibrinolytic agent in the treatment of acute myo-

cardial infarction and pulmonary embolism, both conditions usually resulting from an obstruction of a blood vessel by a fibrin-containing thrombus.

In addition to its striking fibrin specificity, t-PA exhibits several further distinguishing characteristics:

(a) T-PA differs from most serine proteases in that the single-chain form of the molecule has appreciable enzymatic activity. Toward some small substrates, and toward plasminogen in the absence of fibrin, two-chain t-PA has greater activity than one-chain t-PA. In the presence of fibrin, however, the two forms of t-PA are equally active (Rijken et al., *J. Biol. Chem.*, 257: 2920–2925 (1982); Lijnen et al., *Thromb. Haemost.*, 64: 61–68 (1990); Bennett et al., *J. Biol. Chem.*, 266: 5191–5201 (1991)). Most other serine proteases exist as zymogens and require proteolytic cleavage to a two-chain form to release full enzymatic activity.

(b) The action of t-PA in vivo and in vitro can be inhibited by a serpin, PAI-1 (Vaughan et al., *J. Clin. Invest.*, 84: 586–591 (1989); Wiman et al., *J. Biol. Chem.*, 259: 3644–3647 (1984)).

(c) T-PA binds to fibrin in vitro with a K_d in the μM range.

(d) T-PA has a rapid in vivo clearance that is mediated by one or more receptors in the liver (Nilsson et al., *Thromb. Res.*, 39: 511–521 (1985); Bugelski et al., *Throm. Res.*, 53: 287–303 (1989); Morton et al., *J. Biol. Chem.*, 264: 7228–7235 (1989)).

A substantially pure form of t-PA was first produced from a natural source and tested for in vivo activity by Collen et al., U.S. Pat. No. 4,752,603 issued Jun. 21, 1988 (see also Rijken et al., *J. Biol. Chem.*, 256: 7035 (1981)). Pennica et al. (*Nature*, 301: 214 (1983)) determined the DNA sequence oft-PA and deduced the amino acid sequence from this DNA sequence (U.S. Pat. No. 4,766,075 issued Aug. 23, 1988).

Human wild-type t-PA has potential N-linked glycosylation sites at amino acid positions 117, 184, 218, and 448. Recombinant human t-PA (ACTIVASE® t-PA) produced by expression in CHO cells was reported to contain approximately 7% by weight of carbohydrate, consisting of a high-mannose oligosaccharide at position 117, and complex oligosaccharides at Asn-184 and Asn-448 (Vehar et al., "Characterization Studies of Human Tissue Plasminogen Activator produced by Recombinant DNA Technology," *Cold Spring Harbor Symposia on Quantitative Biology*, LI:551–562 (1986)).

Position 218 has not been found to be glycosylated in native t-PA or recombinant wild-type t-PA. Sites 117 and 448 appear always to be glycosylated, while site 184 is thought to be glycosylated only in a fraction of the molecules. The t-PA molecules that are glycosylated at position 184 are termed Type I t-PA, and the molecules that are not glycosylated at position 184 are termed Type II t-PA. In melanoma-derived t-PA, the ratio of Type I to Type II t-PA is about 1:1. The most comprehensive analysis of the carbohydrate structures of CHO cell-derived human t-PA was carried out by Spellman et al., *J. Biol. Chem.*, 264: 14100–14111 (1989), who showed that at least 17 different Asn-linked carbohydrate structures could be detected on the protein. These ranged from the high-mannose structures at position 117 to di-, tri-, and tetra-antennary N-acetyllactosamine-type structures at positions 184 and 448. Type I and Type II t-PAs were reported to be N-glycosylated in an identical way at Asn-117 and Asn-448 positions, when isolated from the same cell line. For further details, see also Parekh et al., Biochemistry, 28: 7644–7662 (1989). The specific fibrinolytic activity of Type II t-PA has been shown to be about 50% greater than that of Type I t-PA

(Einarsson et al., *Biochim. Biophys. Acta*,830: 1–10 (1985)). Further, increased Type I is correlated with increased half-life (Cole et al., *Fibrinolysis*, 7: 15–22 (1993)). However, Type II t-PA, which lacks a portion of carbohydrate associated with Type I t-PA, as well as desialated t-PA, demonstrated a longer T½ beta than standard t-PA (Beebe and Aronson, *Thromb. Res.* 51: 11–22 (1988)).

Analysis of the sequence of t-PA has identified the molecule as having five domains. Each domain has been defined with reference to homologous structural or functional regions in other proteins such as trypsin, chymotrypsin, plasminogen, prothrombin, fibronectin, and epidermal growth factor (EGF). These domains have been designated, starting at the N-terminus of the amino acid sequence of t-PA, as the finger (F) domain from amino acid 1 to about amino acid 44, the growth factor (G) domain from about amino acid 45 to about amino acid 91 (based on homology with EGF), the kringle-1 (K1) domain from about amino acid 92 to about amino acid 173, the kringle-2 (K2) domain from about amino acid 180 to about amino acid 261, and the serine protease (P) domain from about amino acid 264 to the carboxyl terminus at amino acid 527. These domains are situated essentially adjacent to each other, and are connected by short "linker" regions. These linker regions bring the total number of amino acids of the mature polypeptide to 527, although three additional residues (Gly-Ala-Arg) are occasionally found at the amino terminus. This additional tripeptide is generally thought to be the result of incomplete precursor processing, and it is not known to impart functionality. Native t-PA can be cleaved between position 275 and position 276 (located in the serine protease domain) to generate the two-chain form of the molecule.

Each domain contributes in a different way to the overall biologically significant properties of the t-PA molecule. Domain deletion studies show that the loss of the finger, growth factor, or kringle-2 domain results in a lower-affinity binding of the variant t-PA to fibrin (van Zonneveld et al., Proc. Natl. Acad. Sci. USA, 83: 4670–4674 (1986); Verheijen et al., *EMBO J.*, 5: 3525–3530 (1986)); however, more recent results obtained with substitution mutants indicate that the kringle-2 domain is less involved in fibrin binding than earlier expected (Bennett et al., supra). The domain deletion studies have implicated the finger and growth factor domains in clearance by the liver (Collen et al., *Blood*, 71: 216–219 (1988); Kalyan et al., *J. Biol. Chem.*, 263: 3971–3978 (1988); Fu et al., *Thromb. Res.*, 50: 33–41 (1988); Refino et al., *Fibrinolysis*, 2: 30 (1988); Larsen et al., *Blood*, 73: 1842–1850 (1989); Browne et al., *J. Biol. Chem.*, 263: 1599–1602 (1988)). The kringle-2 domain is responsible for binding to lysine. The serine protease domain is responsible for the enzymatic activity of t-PA and contains specific regions where mutations were shown to affect both fibrin binding and fibrin specificity (possibly direct interactions with fibrin), and other regions where only fibrin specificity is altered (possibly indirect interactions with fibrin) (Bennett et al., supra). Studies with mutants resulting from site-directed alterations indicate the involvement of the glycosylation of t-PA in clearance (Lau et al., *Bio/Technology*, 5: 953–958 (1987); Lau et al., *Bio/Technology*, 6: 734 (1988)).

An unglycosylated variant of t-PA consisting of the kringle-2 and protease domains was described to have a slower plasma clearance than wild-type t-PA (Martin et al., *Fibrinolysis*, 4:(Suppl.3):9 (Abstract 26) (1990)). The effects of altering oligosaccharide structures at sites 164 and 448 of t-PA were also examined by Howard et al., *Glycobiology*, 1: 411–418 (1991). Hotchkiss et al. (*Thromb. Haemost.*, 60: 255–261 (1988)) selectively removed oligosaccharide residues from the t-PA molecule, and demonstrated that the removal of these residues decreased the rate of clearance of t-PA. These researchers, and Lau et al. ((1987), supra, (1988), supra) also generated the t-PA variant N117Q (wherein asparagine at position 117 of wild-type human t-PA was substituted with glutamine) to prevent glycosylation at position 117. This variant, similarly to that obtained by enzymatic removal of the high-mannose oligosaccharide at this position, exhibited an about two-fold slower clearance rate than wild-type human t-PA. See also EP-A 238,304 published Sep. 23, 1987 and EP-A 227,462 published Jul. 1, 1987.

Several reports have suggested that the carbohydrate moieties of t-PA influence the in vitro activity of this enzyme (Einarsson et al., supra; Opdenakker et al., *Proc. Sci. Exp. Biol. Med.*, 182: 248–257 (1986)). T-PA is endocytosed by mannose receptors of liver endothelial cells and by galactose receptors of parenchymal cells. Indeed, the in vivo clearance of recombinant human t-PA produced in mammalian cell cultures was influenced by carbohydrate structures, particularly by the high-mannose oligosaccharides (Hotchkiss et al., supra). A t-PA variant (designated TNK t-PA) that has a glycosylation site added at amino acid position 103, the native glycosylation site removed at amino acid position 117, and the sequence at amino acid positions 296–299 of native human t-PA replaced by AAAA, has been shown to have increased circulatory half-life, and markedly better fibrin specificity than wild-type human t-PA (Keyt et al., *Proc. Natl. Acad. Sci. USA*, 91: 3670–3674 (1994)).

Glycoproteins Other than Native t-PA with More than One Glyoform

Cells expressing tPA-6, a molecule composed of the kringle-2 and serine protease domains of t-PA, process it into two glycoforms, a monoglycosylated form with Asn-448 occupied, and a diglycosylated form with Asn-448 and Asn184 occupied (Berg et al., *Blood*, 81: 1312–1322 (1993)).

Plasminogen exists in two glycoforms. The more glycosylated form, commonly referred to as "plasminogen-1," "plasminogen I," or "Type 1 plasminogen," has a galactosamine-based oligosaccharide attached at amino acid position 345 (Thr345) and a complex glycosamine-based oligosaccharide at amino acid position 288 (Asn288) of a native human plasminogen molecule. The less glycosylated form, commonly referred to as "plasminogen-2," "plasminogen II," or "Type 2 plasminogen," has a single oligosaccharide chain attached at amino acid position 345 (Thr345) (Hayes and Castellino, *J. Biol. Chem.*, 254(18): 8772–8776, 8777–8780 (1979); Lijnen et al., *Eur. J. Biochem.*, 120: 149–154 (1981); Takada et al., *Thrombosis Research*, 39: 289–296 (1985)).

Other glycoproteins displaying variable site occupancy (variations in N- and O-glycosylation site-occupancy) include granulocyte-macrophage colony-stimulating factor (Okamoto et al., *Archives of Biochemistry and Biophysics*, 286:562–568 (1991)), interferon-gamma (Curling et al., *Biochem. J.*, 272: 333–337 (1990)), protein C (Miletich and Broze, *J. Biol. Chem.*, 265: 11397–11404 (1990)), and interleukin-2. Glycosylation of gamma-interferon was stable throughout an optimized culture design strategy using fed-batch cultures, with exposure to glucose starvation possibly leading to a dramatic change in glycosylation efficiency (Xie et al., *Biotechnol. Bioeng.*, 56: 577–582 (1997)).

Different factors have been discussed to be potentially responsible for variable site-occupancy, including availability of dolichol-phosphate and nucleotide sugars (Nyberg et

al., *Biotechnol. Bioeng.*, 62: 336–347 (1999)), glycosyltransferase activity (Hendrickson and Imperiali, *Biochemistry*, 34: 9444–9450 (1995); Kaufman et al., *Biochemistry*, 33: 9813–9819 (1994)), and variable glycosylation site accessibility due to competition with protein folding (Holst et al., *The EMBO J.*, 15: 3538–3546 (1996); Imperiali, *Acc. Chem. Res.*, 30: 452–459 (1997); Shelikoff et al., *Biotechnol. Bioeng.*, 50: 73–90 (1996)). Any of these factors could be influenced by cell culture conditions. T-PA site-occupancy usually varies within a rather narrow range (±5%).

Studies of Oligosaccharyltransferase/Metal-gations in Glycosylation

Asparagine-linked glycosylation involves the enzyme-catalyzed modification of an asparagine side chain in a nascent polypeptide with a tri-antennary tetradecasaccharide moiety. This first committed step in the biosynthesis of N-linked glycoproteins is catalyzed by oligosaccharyltransferase, a heteromeric membrane-associated enzyme complex found in the lumen of the endoplasmic reticulum of eukaryotic cells. See Imperiali, supra; Allen et al., *J. Biol. Chem.*, 270: 4797–4804 (1995); Sharma et al., *Eur. J. Biochem.*, 116: 101–108 (1981); Silberstein and Gilmore, *The FASEB Journal*, 10: 849–858 (1996); Kumar el al., *Biochem. Mol. Biol. Intl.*, 36: 817–826 (1995); Bause et al., *Biochem. J.*, 312: 979–985 (1995); Xu and Coward, *Biochemistry*, 36: 14683–14689 (1997); Kumar et al., *Biochem. Biophys. Res. Comm.*, 247: 524–529 (1998); Watt et al., *Curr. Op. Struct. Biol.*, 7: 652–660 (1997).

For optimal activity, oligosaccharyltransferase requires a small amount of manganese divalent ion, but other divalent metal cations with an octahedral coordination geometry will support transfer, although at reduced rates (Hendrickson and Imperiali, supra; Kaufman et al., supra; Kumar et al., *Biochem. & Mol. Biol. International*, 36: 817–826 (1995)).

The Role of Temperature in Mammalian Cell Cultures

To simulate normal body environment, fermentor temperature in cultivating mammalian cells is controlled almost exclusively at 37° C. This dogma is so widely accepted that, so far, little attention has been paid to varying temperature in the cell culture process. The scarce literature data suggest that reduced fermentor temperature results in improved cell viability and shear resistance, higher cell density and titer in batch cultures, and a reduction in glucose/lactate metabolism (Chuppa et al., *Biotechnol. Bioeng.*, 55: 328–338 (1997)).

Specifically, Reuveny et al., *J. Immunol. Methods*, 86: 53–59 (1986) studied the effect of temperatures in the range of 28° C. to 37° C. on batch hybridoma cell cultures. They found that although at lower temperatures the cell viability was improved, this was accompanied by a decrease in glucose uptake and a decrease in the specific antibody production. Therefore, in this particular case, lower temperatures did not enhance the overall performance of the cell culture process.

Sureshkumar and Mutharasan, *Biotechnol. Bioeng.*, 37: 292–295 (1991) investigated the effect of the temperature range of 29° C. to 42° C. on the cell culture process, and found that maximum cell density was achieved at 33° C. In contrast, the glucose uptake and specific lactate production rates were dramatically lower at 33° C. than at 39° C. These results showed that the optimal temperatures for growth and productivity may considerably differ. While the viability increase at temperatures below 37° C. appears to be a general phenomenon, the effect of temperature on specific productivity has been shown to be cell-line dependent (Chuppa et al., supra).

Weidemann et al., *Cytotechnology*, 15: 111–116 (1994) cultivated adherent recombinant baby hamster kidney (BHK) cells at temperatures between 30° and 37° C. The low-temperature cultivation in batch and repeated batch mode in a two-liter bioreactor showed a lower growth rate and a lower glucose consumption rate (i.e., less lactate production). On the other hand, the maximum cell density and productivity were not affected by the temperature reduction.

Kretzmer et al., "Cultivation Temperature-Effect on Cell Culture Processes and Their Optimization" (American Chemical Society Meeting, San Francisco, Calif.), abstract 138, presented Apr. 16, 1997, disclosed the effect of cultivation temperature on cell culture processes and their optimization, but apparently no specific glycosylation analysis.

It has been suggested that reduced fermentor temperatures might have other advantages related to product quality and integrity, but the effect of low temperatures on product quality, and in particular, on protein glycosylation, has been scarcely studied. Chuppa et al., supra, have reported that fermentation temperature did not have a significant effect on the sialic acid content of glycoproteins. Although the total sugar content was somewhat lower at 37° C. than at 34° C. or 35.5° C., the authors viewed this difference as "not substantial."

However, U.S. Pat. No. 5,705,364 described preparing glycoproteins by mammmalian cell culture wherein the sialic acid content of the glycoprotein produced was controlled over a broad range of values by manipulating the cell culture environment, including the temperature. The host cell was cultured in a production phase of the culture by adding an alkanoic acid or salt thereof to the culture at a certain concentration range, maintaining the osmolality of the culture at about 250 to about 600 mOsm, and maintaining the temperature of the culture between about 30 and 35° C.

Bahr-Davidson, "Factors Affecting Glycosylation Site Occupancy of ASN- 184 of Tissue-Type Plasminogen Activator Produced in Chinese Hamster Ovary Cells," A Dissertation submitted to the Department of Chemical Engineering and the Committee of Graduate Studies of Stanford University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy, May 1995, investigated the effects of temperature on glycosylation site occupancy and reported that site occupancy was increased by exposing cells to 26° C. (see pages 50–51).

Hormonal and Other Treatments to Influence Glycosylation

The effect of various additives such as components of plasma to the culture media on protein production and glycosylation has been studied in the literature, for example, the effects of hormonal treatments on membrane glycosylation in rat kidney brush broder membranes (Mittal et al., *Indian J. Exper. Biol.*, 34: 782–785 (1996)). Studies of Muc-1 mucin expression established the hormonal basis for MRNA expression (Parry et al., *J. Cell Sci.*, 101: 191–199 (1992)). Thyroid hormone regulation of alpha-lactalbumin with differential glycosylation has been reported (Ziska et al., *Endocrinology*, 123: 2242–2248 (1988)). The cellular response to protein N-glycosylation was increased in the presence of thyroxine, insulin, and thrombin, and the effect was dose-dependent (Oliveira and Banerjee, *J. Cell. Physiol.*, 144: 467–472 (1990)). Thyroxine was found to induce changes in the glycosylation pattern of rat alpha-fetoprotein (Naval et al., *Int. J. Biochem.*, 18: 115–122 (1986)).

In addition to hormonal treatments, glutatb one and glucose-6-phosphate dehydrogenase deficiency increased

protein glycosylation (Jain, *Free Radical Biology & Medicine,* 24: 197–201 (1998)). Thyrotropin was found to control oligosaccharyltransferase activity in thyroid cells (Desruisseau et al., *Mol. Cell. Endocrinol.,* 122: 223–228 (1996)). The addition of glucose and tri-iodothyronine (T_3) to a medium producing a pro-urokinase derivative improved productivity (Hosoi et al., *Cytotechnoloy,* 19: 1–10 (1996)). Also, fucosyltransferase activity in the rat small intestine was responsive to hydrocortisone regulation during the suckling period (Biol et al., *Biochim. Biophys. Acta,* 1133: 206–212 (1992)). Hydrocortisone treatment also induced quantitative alterations in glycosylation of mouse mammary tumor virus precursors (Maldarelli and Yagi, *JNCI,* 77: 1109–1115 (1986)). Glycosylation of cellular glycoconjugates in a carcinoma cell line was enhanced by a retinoic acid (Sacks et al., *Glycoconjugate J.,* 13: 791–796 (1996)). Further, retinoic acid had reversible effects on glycosaminoglycan synthesis during differentiation of HL-60 leukemia cells (Reiss et al., *Can Res.,* 45: 2092–2097 (1985)). Additionally, retinoic acid, as well as hydrocortisone, was found to modulate glycosaminoglycan synthesis of human malignant keratinocytes (Reiss et al., *J. Invest. Dermatol.,* 86: 683–688 (1986)).

The competition between folding and glycosylation in the endoplasmic reticulum has been described (Holst et al., supra), as has acute heat shock inducing the phenomenon of prompt glycosylation (Jethmalani et al., *J. Biol. Chem.,* 269: 23603–23609 (1994)).

There is a need for increasing glycosylation site occupancy in glycoproteins having multiple glycoforms to produce glycoprotein therapeutics of consistent product quality. For example, there is a need to increase the fraction of Type I t-PA in the t-PA production process. Such increase in site-occupancy generates t-PA with activity more closely resembling the international human t-PA standard, and thus more closely resembling human t-PA. Type I t-PA is also more soluble than Type II, which may be of some value in processing steps. Further, increased Type I is correlated with increasing circulatory half-life, as noted above.

SUMMARY OF THE INVENTION

It has been found that during the production of a wild-type glycoprotein, namely human t-PA, in mammalian cells, namely Chinese Hamster Ovary (CHO) cells, use of certain divalent metals, hormones, or factors that manipulate cell-cycle distribution to control or influence glycosylation significantly increases site occupancy of a glycosylation site of the glycoprotein. For example, decreasing the cultivation temperature from 37° C. to about 30–35° C. in the production phase significantly enhances the occupancy of the glycosylation site at amino acid position 184, and thereby increases the ratio of Type I t-PA to Type II t-PA. Specifically, decreasing the temperature from 37 to 33 or 31° C. increased t-PA site-occupancy up to 6%. Temperatures below 37° C. are expected similarly to facilitate the occupancy of not-easily-accessible N-linked glycosylation sites in other glycoproteins. Accordingly, temperature can be used as a sensitive tool for fine tuning the ratio of variously glycosylated forms of glycoproteins having one or more N-linked glycosylation sites occupied only in a fraction of the protein.

In addition, other environmental factors, including those that manipulate the culture's growth state, and correspondingly cell-cycle distribution, such as butyrate, or a cell-cycle inhibitor that increases the proportion of cells in the G0/G1 phase such as quinidine, plasma components such as thyroid hormones, and/or certain divalent metal cations significantly elevated the t-PA Type I content (about 1–2.5%) compared to control conditions, and are expected to act similarly with respect to other glycoproteins. In contrast, addition of the relevant nucleoside precursor molecules (e.g., uridine, guanosine, mannose) did not result in improved site-occupancy.

In one aspect, the invention concerns a process for producing a glycoprotein comprising culturing mammalian host cells producing the glycoproteins (i.e., cells expressing nucleic acid encoding the glycoprotein) in the presence of (a) a factor that modifies growth state in a cell culture, (b) a divalent metal cation that can adopt and prefers an octahedral coordination geometry, or (c) a plasma component, whereby the occupancy of an N-linked glycosylation site occupied only in a fraction of the glycoprotein is enhanced in the glycoprotein so produced. Preferably, the factor is a cell-cycle inhibitor that blocks cells in the G0/G1 phase, a butyrate salt, and/or a temperature of the culture of between about 30 and 35° C., the divalent cation is manganese or iron, and the plasma component is a hormone. Preferably, the cell culture procedure includes a growth phase, followed by a transition phase and a production phase. In a preferred embodiment, in the growth phase the mammalian host cells are cultured at about 37° C., whereupon, during the transition phase, the temperature is lowered to between about 30° C. and 35° C. The host cells preferably are CHO cells, and the glycoprotein preferably is t-PA.

In another aspect, the invention provides a process for producing human t-PA comprising culturing CHO cells expressing nucleic acid encoding said t-PA in a serum-free medium in a production phase at a temperature of between about 30° C. and 35° C. and in the presence of about 0 to 2 mM of a butyrate salt, whereby the occupancy of an N-linked glycosylation site occupied only in a fraction of t-PA is enhanced in the t-PA so produced.

In a still further aspect, the invention supplies a process for producing human t-PA comprising culturing CHO cells expressing nucleic acid encoding said t-PA in a serum-free medium in a growth phase at a temperature of about 37–40° C., wherein said medium comprises from about 10 nM to 100 μM of adivalent metal cation that can adopt and prefers an octahedral coordination geometry; culturing said cell in a transition phase at a temperature of about 37–40° C.; and culturing said cell in a production phase wherein after about 48 hours into the production phase the temperature is lowered to between about 30° C. and 35° C. and about 0.75 to 1.5 mM of a butyrate salt is added to the medium, whereby the occupancy of an N-linked glycosylation site occupied only in a fraction of t-PA is enhanced in the t-PA so produced. In this process, a plasma component such as a thyroid hormone, e.g., thyroxine or tri-iodothyronine, or a cell-cycle inhibitor that blocks cells in the G0/G1 phase such as quinidine is optionally added to the culture medium before or during the growth phase.

Hence, the process herein facilitates the production of a preferred glycoform of a glycoprotein, such as Type I t-PA, in a mammalian cell culture, and also increases the ratio of preferred to non-preferred glycoproteins, such as the ratio of Type I to Type II t-PA, in a mammalian cell culture.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows a depiction of Type I t-PA and Type II t-PA and a chromatogram thereof.

FIG. **2** shows the percentage of Type I human t-PA in cell cultures of CHO cells cultivated at various scales at 37° C.

FIG. **3** shows graphs of the percentage of Type I human t-PA in cell cultures of CHO cells at 37° C. as a function of

12K-fermentation run time, with each graph line representing a different run.

FIGS. **4**A and **4**B show the percentage of Type I human t-PA in cell cultures of CHO cells cultivated on a laboratory scale (in 25 cm^2 T-flasks and 100-ml spinner flasks-FIG. **4**A) or in a 5-liter bioreactor (FIG. **4**B for 5–7 days at 33° C. relative to control (cell culture kept at 37° C.).

FIG. **5** shows the percentage of Type I human t-PA in cell cultures of CHO cells cultivated in T-flasks at 37° C. for 34 days wherein sodium butyrate is added in the amount indicated at the time of inoculation. The values are from triplicate experiments.

FIG. **6** shows the percentage of Type I human t-PA in cell cultures of CHO cells cultivated in 60-mm culture dishes at 37° C. for 4–6 days wherein temperature changes with or without sodium butyrate are compared (wherein 37° C.1—means control at 37° C. with no butyrate; 37° C.11× is at 37° C. with 0.75 mM butyrate, 37° C.12× is at 37 ° C. with 1.5 mM butyrate, 33° C.1—is at 33° C. with no butyrate, 33° C.11× is at 33° C. with 0.75 mM butyrate, 33° C.12× is at 33° C. with 1.5 mM butyrate, 31° C.1—is at 31° C. with no butyrate, 31° C.11× is at 31° C. with 0.75 mM butyrate, and 31° C.12× is at 31° C. with 1.5 mM butyrate).

FIG. **7** shows percentage of Type I human t-PA in cell cultures of CHO cells cultivated in 5-liter bioreactors after 5–7 days, wherein temperature changes with or without sodium butyrate are compared (where control is at 37° C. without butyrate, 33° C. is at 33° C. without butyrate, 2× butr. is at 37° C. with 1.5 mM butyrate, and 33° C./2× is at 33° C. with 1.5 mM butyrate).

FIG. **8** shows the percentage of Type I human t-PA in cell cultures of CHO cells cultivated in fermentors over time at 37° C. in which sodium butyrate was added to a concentration of 0.75 mM at approximately 48 hours, and the percentages of cells in G0/G1 phase at the corresponding time points. These results reflect the averages of three separate cultures.

FIG. **9** shows the percentage of Type I human t-PA in cell cultures of CHO cells cultivated in T-flasks at 37° C. for 3–4 days wherein at the time of inoculation no cell-cycle inhibitor is added (control), thymidine (250 μg/mL) is added, or quinidine (90 μM) is added. The values are from triplicate experiments.

FIGS. **10**A and **10**B show the percentage of Type I human t-PA in cell cultures of CHO cells cultivated in 60-mm culture dishes at 37° C. for 4–6 days wherein at the time of inoculation 3 nM of $MnCl_2$ is added (control), or 10 nM, 100 nM, 1 μM, 10 μM, or 100 μM $MnCl_2$ is added. The values are expressed in triplicate for FIG. **10**A and as an average of triplicates for FIG. **10**B.

FIG. **11** shows the percentage of Type I human t-PA in cell cultures of CHO cells cultivated in 60-mm culture dishes at 37° C. for 4–6 days wherein at the time of inoculation no ferric citrate is added (control), or 10 μM ferric citrate, 50 μM ferric citrate, or 100 μM ferric citrate is added.

FIG. **12** shows the percentage of Type I human t-PA in cell cultures of CHO cells cultivated in 60-mm culture dishes at 37° C. for 4–6 days wherein the cells are cultivated in the presence of increased amounts of specified nucleotide sugar precursor molecules.

FIGS. **13**A and **13**B show the percentage of Type I human t-PA in cell cultures of CHO cells cultivated in 60-mm culture dishes at 37° C. for 7 days wherein at the time of inoculation no hormone is added (control), or 1 nM, 10 nM, or 100 nM of tri-iodothyronine (Triiod.) or of thyroxine (Thyrox.) is added. The values are expressed in triplicate for FIG. **13**A and as an average of triplicates for FIG. **13**B.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

As used herein, the word "enhanced" as it relates to occupancy of an N-linked glycosylation site occupied only in a fraction of the glycoprotein refers to relative value obtained by practicing the current invention versus control value obtained by not using the parameters of this invention. The value is calculated based on the percentage of glycosylation sites occupied at the particular position of the glycoprotein in question versus a baseline value, which is determined without using the factors, cations, or plasma components herein claimed. For example, t-PA is secreted as a mixture of two major glycoforms, Type I (all three N-glycosylation sites are occupied) and Type II (Asn-184 is not occupied), and an enhanced occupancy level means an increased site occupancy such that the mixture has increased amounts of Type I relative to Type II t-PA versus the control. This occupancy level can be measured, for example, by using reversed-phase high-pressure liquid chromatography (RP-HPLC) to elute the fragments of the different types of glycoprotein (the types having different glycosylation site occupancy levels) and integrating the peak areas for each type of glycoprotein to determine relative quantities. The most typical way to express these quantities is by the percentage of the higher-occupancy type of glycoprotein to total types of glycoprotein. A specific example of an assay used to measure enhancement for Type I/Type II t-PA is set forth below in Example 1.

As used herein, "glycoprotein" refers generally to peptides and proteins having more than about ten amino acids and at least one glycosylation site that is occupied only in a fraction of the glycoprotein product, i.e., they display variable site-occupancy or variations in N- and O-glycosylation site-occupancy. The glycoproteins may be homologous to the host cell, or preferably, they are heterologous, i.e., foreign, to the host cell being utilized, such as a human protein produced by a CHO cell. Preferably, mammalian glycoproteins (glycoproteins that were originally derived from a mammalian organism) are used, more preferably, those which are directly secreted into the medium, and most preferably, those wherein N-glycosylation site-occupancy is involved.

The specifically-preferred glycoproteins herein are t-PA, plasminogen, interferon-gamma, Protein C, IL-2, and CSF, for example, GM-CSF. The more preferred glycoproteins are t-PA or plasminogen, and the most preferred is t-PA, more notably human t-PA.

The terms "tissue plasminogen activator", and "t-PA" refer to human extrinsic (tissue-type) plasminogen activator having fibrinolytic activity that typically has a structure with five domains (finger, growth factor, kringle-1, kringle-2, and protease domains), but nonetheless may have fewer domains or may have some of its domains repeated if it still functions as a thrombolytic agent and retains the N-linked glycosylation sites at positions 117, 184, and 448. At minimum, the t-PA consists of a protease domain that is capable of converting plasminogen to plasmin, and an N-terminal region believed to be at least partially responsible for fibrin binding, and retains the N-linked glycosylation sites at positions corresponding to amino acid positions 117, 184, and 448 of wild-type human t-PA. The retention of these glycosylation sites is due to the fact that variable site occupancy of recombinant and melanoma-derived wild-type t-PA leads to production of two variants, designated as "Type I t-PA" and

"Type II t-PA", respectively. Type I t-PA contains N-linked oligosaccharides at positions 117, 184, and 448. Type II t-PA contained N-linked oligosaccharides at positions 117 and 448. See FIG. 1. It will be understood that natural allelic variations exist and can occur among individuals, as demonstrated by one or more amino acid differences in the amino acid sequence of t-PA of each individual.

The terms "wild-type human tissue plasminogen activator", "wild-type human t-PA", "native human tissue plasminogen activator," and "native human t-PA", where "human t-PA" may be abbreviated as "ht-PA", refer to native-sequence human t-PA, i.e., that encoded by the cDNA sequence reported in U.S. Pat. No. 4,766,075, issued Aug. 23, 1988. Amino acid site numbers or positions in the t-PA molecule are labeled in accordance with U.S. Pat. No. 4,766,075. The t-PA may be from any native source. In addition, the t-PA may be obtained from any recombinant expression system, including, for example, CHO cells or human embryonic kidney 293 cells.

As used herein, references to various domains of t-PA mean the domains of wild-type human t-PA as hereinabove defined, and functionally equivalent portions of human t-PA having amino acid alterations as compared to the native human t-PA sequence, or of (native or variant) t-PA from other sources, such as bat tissue plasminogen activator (bat-PA). Thus, as used herein, the term "protease domain" refers to the region extending from amino acid position 264 to amino acid position 527, inclusive, of the mature form of wild-type human t-PA, and to functionally equivalent portions of human t-PA having amino acid alterations as compared to the native human t-PA sequence, or of t-PA from other sources, such as bat-PA.

As used herein, "factor that modifies growth state in a cell culture" refers to a factor that increases the proportion of cells in the G0/G1 phase of growth such as a cell-cycle inhibitor that causes cells to accumulate or blocks the cells in the G0/G1 phase. Such factors manipulate cell cycle distribution to control or influence glycosylation. Such a factor may affect glycosylation in mechanisms beyond growth state, but are defined herein as affecting at least the growth state.

As used herein, a "cell-cycle inhibitor that blocks cells in the G0/G1 phase of growth" is a molecule that causes cells to accumulate in the G0/G1 phase of growth. This can be determined by cell cycle analysis, i.e., uniform suspensions of nuclei are stained with propidium iodide (PI) using the detergent-trypsin method of Vindelov et al., *Cytometry,* 3: 323–327 (1983) to determine relative cellular DNA content by flow cytometric analysis. Events are gated using doublet discrimination to exclude doublets, and the data are modeled using ModFit LT Cell Cycle Analysis™ software (Verity Software House). A preferred such inhibitor herein is quinidine.

By "butyrate" or "butyrate salt" is meant any corresponding salt of butyric acid, such as sodium butyrate or potassium butyrate.

By "phase" is meant a certain phase of culturing of the cells as is well recognized by the practitioner. For example, "growth phase" of the cell culture refers to the period of exponential cell growth (the log phase) where cells are generally rapidly dividing. During this phase, cells are cultured for a period of time, usually between 1–4 days, and under such conditions that cell growth is maximized. The growth cycle for the host cell can be determined for the particular host cell envisioned without undue experimentation. During the growth phase, cells are cultured in nutrient medium containing the necessary additives generally at about 30-40° C., preferably about 37° C., in a humidified, controlled atmosphere, such that optimal growth is achieved for the particular cell line. Cells are maintained in the growth phase for a period of between about one and four days, usually between about two and three days.

"Transition phase" of the cell culture refers to the period of time during which culture conditions for the production phase are engaged. During the transition phase environmental factors such as temperature are shifted from growth conditions to production conditions.

"Production phase" of the cell culture refers to the period of time during which cell growth has plateaued. During the production phase, logarithmic cell growth has ended and glycoprotein production is primary. During this period of time the medium is generally supplemented to support continued glycoprotein production and to achieve the desired glycoprotein product.

By "divalent metal cation that can adopt and prefers an octahedral coordination geometry" is meant a metal cation with two valencies that is capable of, and actually shows preference for, adopting an octahedral coordination geometry. Such cations are also characterized in that oligosaccharyltransferase can function (i.e., is activated) in their presence. Examples of such metal ions include manganese (Mn^{2+}), iron (Fe^{2+}), calcium (Ca^{2+}), and magnesium (Mg^{2+}). Divalent cations that show preferences for other coordination geometries, including nickel (Ni^{2+}), copper (Cu^{2+}), cadmium (Cd^{2+}), and zinc (Zn^{2+}), fail to activate the enzyme and at high concentrations also competitively inhibit activity in the presence of manganese. Hence, these latter cations are excluded from the definition.

By "plasma component" is meant a constituent of normal plasma. This would include growth promoters and tumor-promoting agents for endothelial cell growth, regulators of differentiation of epithelial tissues, glucagon, heparin, phorbol myristate acetate, PRL, thyroglobulin, 8Br-cAMP, thrombin, vitamin A and its derivatives (retinoids such as retinoic acid, e.g., beta-all-trans retinoic acid), glutathione, steroids such as corticosterone, cortisol, and corticoids, e.g., glucocorticoids such as hydrocortisone, and hormones, preferably those that are vital hormones of metabolism such as estrogen, insulin, and thyroid hormones, e.g., thyroxine and tri-iodothyronine (T_3). The thyroid hormones are preferred, and most preferably thyroxine and tri-iodothyronine. Since some serum, including fetal calf serum, contains thyroid hormones and the thyroid hormone binding protein at nanomolar levels, it is preferred to use serum-free medium, particularly if thyroid hormones are employed to enhance site-occupancy.

The terms "cell culture medium," "culture medium," and "fermentation medium" refer to a nutrient solution used for growing mammalian cells that typically provides at least one component from one or more of the following categories:

1) an energy source, usually in the form of a carbohydrate such as glucose;
2) all essential amino acids, and usually the basic set of twenty amino acids plus cysteine;
3) vitamins and/or other organic compounds required at low concentrations;
4) free fatty acids; and
5) trace elements, where trace elements are defined as inorganic compounds or naturally-occurring elements that are typically required at very low concentrations, usually in the micromolar range.

The cell culture medium is generally "serum free" when the medium is essentially free of serum from any mamma-

lian source (e.g. fetal bovine serum (FBS)). By "essentially free" is meant that the cell culture medium comprises between about 0–5% serum, preferably between about 0–1% serum, and most preferably between about 0–0.1% serum. Advantageously, serum-free "defined" medium can be used, wherein the identity and concentration of each of the components in the medium is known (i.e., an undefined component such as bovine pituitary extract (BPE) is not present in the culture medium).

In the context of the present invention the expressions "cell", "cell line", and "cell culture" are used interchangeably, and all such designations include progeny. Thus, the words "transformants" and "transformed (host) cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

The term "mammalian host cell", "host cell", "mammalian cell", "mammalian recombinant host cell," and the like, refer to cell lines derived from mammals that are capable of growth and survival when placed in either monolayer culture or in suspension culture in a medium containing the appropriate nutrients and growth factors. The necessary growth factors for a particular cell line are readily determined empirically without undue experimentation, as described, for example, in *Mammalian Cell Culture,* Mather, J. P. ed. (Plenum Press: N.Y., 1984), and Barnes and Sato, *Cell,* 22: 649 (1980). Typically, the cells are capable of expressing and secreting large quantities of a particular glycoprotein of interest into the culture medium. Examples of suitable mammalian host cells within the context of the present invention may include CHO cells (EP 117,159, published Aug. 29, 1989; U.S. Pat. Nos. 4,766,075; 4,853,330; 5,185, 259; Lubiniecki et al., in *Advances in Animal Cell Biology and Technology for Bioprocesses,* Spier et al., eds. (1989), pp.442–451), for example, CHO derivatives such as CHO/-DHFR (Urlaub and Chasin, *Proc. Nati. Acad. Sci. USA,* 77: 4216 (1980)), CHO-K1 DUX B11 (Simonsen and Levinson, *Proc. Natl. Acad. Sci. USA* 80: 2495–2499(1983); Urlaub and Chasin, supra), and dp 12.CHO cells (EP 307,247 published Mar. 15, 1989); rat myeloma YB2/3.oAg20(WO 86/00127 published Apr. 1, 1985); mouse C127 fibroblasts (Reddy et al., DNA, 6: 461–472 (1987)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.,* 23: 243–251 (1980)); human cervical carcinoma cells (HELA, ATCC CCL 2); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.,* 383: 44–68 (1982)); MRC 5 cells; FS4 cells; and human melanoma cells (Browne et al., *Thromb. Haemost.,* 54: 422–424 (1985)). Preferred host cells include CHO-K1 DUX B11 and dp 12.CHO cells.

The CHO cells developed for large-scale production of t-PA are maintained cryogenically in a MCB/working cell bank (WCB) system as described by Wiebe et al., in *Large Scale Mammalian Cell Culture Technology,* Lubiniecki, ed., (Marcel Dekker: New York, 1990), pp. 147–160. DHFR+ CHO-K1 cells transfected with DNA encoding human t-PA have been deposited at the American Type Culture Collection, Manassas, Va. (ATCC), and are available under accession number CCL 61. A sample of another t-PA-producing CHO cell line (CHO cell line $1–15_{15}$) has been deposited under ATCC accession number CRL 9606. The latter cell line was reported to result in human t-PA levels approaching 50 pg/cell/day.

II. Modes for Carrying Out the Invention

General and Specific Features of Invention

It has been discovered that utilizing a factor that modifies growth state in a cell culture (such as a cell-cycle inhibitor, a butyrate salt, or lowering the temperature during the production of a glycoprotein in a mammalian cell culture from 37° C. to about 30–35° C.), or utilizing a divalent metal cation that can adopt and prefers an octahedral coordination geometry, or utilizing a plasma component, enhances the occupancy of the glycosylation site at a selected and desired amino acid position of the wild-type glycoprotein.

For example, this lowering of temperature enhances the occupancy of the glycosylation site at amino acid position 184 of wild-type human t-PA and, accordingly, increases the ratio of Type I t-PA to Type II t-PA. The ability to adjust, and increase, the Type I to Type II t-PA ratio is significant, since it enables the manufacturer to produce a recombinant protein in which this ratio closely approximates the ratio present in native t-PA (about 1:1). In addition, the ratio of Type I to Type II t-PA affects the solubility and clearance rate of t-PA, and there is evidence that an increased Type I t-PA concentration somewhat increases the circulatory half-life of t-PA. It is known that the high-mannose oligosaccharide at amino acid position 117 is primarily responsible for the rapid clearance of native human t-PA. When this oligosaccharide is removed, it has been observed that Type I t-PA has a longer half-life than Type II t-PA, indicating that there is a secondary mechanism on which the extra oligosaccharide present on Type I t-PA has a positive effect. The experimental findings herein can be extended to other glycoproteins that (like t-PA) have at least one glycosylation site that is occupied only in a fraction of the glycoprotein product.

If the factor is a butyrate salt, generally the butyrate is present in a concentration of up to about 2 mM, more preferably about 0.35 to 2 mM, still more preferably about 0.75 to 1.5 mM. The concentration thereof to be selected within this range depends mainly on the temperature to which the culture is subjected and the type of glycoprotein. Hence, if for t-PA the temperature remains at about 37 ° C. or is lowered to about 33–35° C., the butyrate concentration is preferably lower than about 1.5 mM, and more preferably is about 0.3 to 1 mM, most preferably 0.75 mM. However, if for t-PA the temperature is lowered to about 30–31° C., preferably the butyrate concentration is about 1–2 mM, more preferably about 1.5 mM. This illustration shows that more than one of these factors may be operating or present in the cell culture.

In a preferred aspect, the temperature lowering and/or butyrate addition take(s) place during the production phase of the growth cycle. In such a scenario, the temperature is lowered to between about 30 and 35° C. and/or a butyrate salt is added about 48 hours into the production phase. The production phase is suitably preceded by a growth phase and a transition phase of growth cycle. During the growth phase the temperature is preferably kept at about 37° C., and/or during the transition phase the temperature of the culture is preferably lowered to between about 30° C. and 35° C., more preferably about 31–33° C., and most preferably about 31° C.

Alternatively, or additionally to the factor(s) above, the cells are cultured in the presence of a divalent cation as defined above. The choice of divalent cation to use, as well as the specific concentration thereof, depends, inter alia, on the type of glycoprotein being produced and the metal cations and other components already present in the culture

medium and their respective concentrations. For example, if the glycoprotein has a number of thio groups, it is preferred to use a thiophilic metal such as manganese and iron, with iron being the most thiophilic metal. In contrast, if the glycoprotein contains more oxygen groups, then the oxophilic cations, magnesium and calcium, are preferred. If calcium ion is already present in sufficient quantities in the medium, it is not typically used for the purposes herein and a different metal cation is used. Further, the size of the metal cation may have an influence, with iron and magnesium being smaller and calcium and manganese being larger. Steric effects due to sulfur groups on the glycoprotein may dictate a cation of smaller ionic radius. The preferred divalent metal cation herein is manganese, magnesium, or iron, more preferably manganese or iron, and most preferably manganese.

The divalent metal cation is preferably present in the culture medium during the whole cultivation time, and at least is added during the growth phase. The concentration thereof generally ranges between about 10 nm and 150 μM, preferably from about 10 nm to 100 μM for manganese and from about 20 μM to 100 μM for iron.

In another alternative, alone or together with the divalent cation and/or factor, a plasma component is present during the culturing. The plasma component is typically present in an amount from about 1 nM to 15–20 μM, depending mainly on the type of glycoprotein being produced, the type of plasma component utilized, and the scale of fermentation. For example, if the plasma component is a thyroid hormone, it is preferably present in an amount of about 1–150 nM, preferably about 10–100 nM. If the plasma component is glutathione, it is preferably present at about 1–10 μM, and if hydrocortisone, it is preferably present at about 5–15 nM, preferably about 10 nM. Preferably, the plasma component is a hormone, more preferably a thyroid hormone, and most preferably thyroxine or tri-iodothyronine.

The degree of site-occupancy to be achieved for the glycoprotein is balanced against the desired degree of secretion of the glycoprotein, which is generally taken into account in selecting which factors and other components to employ, and at what concentrations or temperatures. For example, site-occupancy is generally controlled in a select range of about ±5% without affecting t-PA secretion.

Cell Culture Procedures

According to the present invention mammalian cells are cultured to produce a desired glycoprotein product. In choosing a host cell for the production of the glycoprotein within the context of the present invention, it is important to recognize that different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage) of the proteins expressed. Appropriate cell lines should be chosen to ensure that the desired post- translational modifications are possible. Alternatively, host cells can be modified to express a desired gene product required for the specific post-translational modification.

In particular, the mammalian cells that express the desired glycoprotein should express or be manipulated to express the particular enzymes such that under suitable conditions, the appropriate post-translational modification occurs in vivo. The enzymes include those enzymes necessary for the addition and completion of N- and O-linked carbohydrates such as those described in Hubbard and Ivatt, *Ann., Rev. Biochem.*, 50: 555–583 (1981) for N-linked oligosaccharides. The enzymes optionally include oligosaccharyltransferase, alpha-glucosidase I, alpha-glucosidase II, ER alpha(1,2)mannosidase, Golgi alpha-mannodase I, N-acetylyglucosaminyltransferase I, Golgi alpha-mannodase II, N-acetylyglucosaminyltransferase II, alpha(1 ,6)fucosyltransferase, β(1,4)galactosyltransferase, and an appropriate sialyltransferase.

For culturing the mammalian cells that express the desired glycoprotein and are capable of adding the desired carbohydrates in specific position and linkage, numerous culture conditions can be used, paying particular attention to the host cell being cultured. Suitable culture conditions for mammalian cells are well known in the art (Cleveland et al., *J. Immunol. Methods,* 56: 221–234 (1983)) or can be easily determined by the skilled artisan (see, for example, *Animal Cell Culture: A Practical Aprroach* 2nd Ed, Rickwood, D. and Hames, B. D., eds. (Oxford University Press: New York, 1992)), and vary according to the particular host cell selected.

The mammalian cell culture of the present invention is prepared in a medium suitable for the particular cell being cultured. The nutrient solution may optionally be supplemented with one or more components from any of the following categories:

1) plasma components as defined above and/or growth factors such as, for example, insulin, transferrin, and EGF;
2) salts and buffers such as, for example, sodium chloride, calcium, magnesium, phosphate, and HEPES;
3) nucleosides and bases such as, for example, adenosine, thymidine, and hypoxanthine;
4) protein and tissue hydrolysates;
5) antibiotics such as GENTAMYCIN™ drug; and
6) lipids such as linoleic or other fatty acids and their suitable carriers.

Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art.

Commercially available media such Ham's F10 (Sigma), Minimal Essential Medium (MEM, Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM, Sigma) are exemplary nutrient solutions. In addition, any of the media described in Ham and McKeehan, *Meth. Enz.*, 58: 44 (1979); Barnes and Sato, *Anal. Biochem.*, 102: 255 (1980); U.S. Pat. No. 4,767,704; U.S. Pat. No. 4,657,866; U.S. Pat. No. 4,927,762; U.S. Pat. No. 5,122,469 or U.S. Pat. No. 4,560,655; WO 90/03430; and WO 87/00195 may be used as culture media. Any of these media may be supplemented as necessary with the components as mentioned above.

The use of a special medium lacking animal serum (serum-free medium) is preferred to avoid interference or counter-action from components of the serum with one or more of the factors, divalent metal cations, plasma components, or other ingredients employed in accordance with the present invention. Moreover, the concentration of the amine groups should be sufficiently high to keep t-PA in solution as the concentration increases. This can be achieved by using greater than about 1 mM lysine concentrations, by the presence of HEPES, or by the use of sufficiently high ammonium chloride concentrations, although any other amine or ammonium source will do.

If the goal is to produce t-PA in substantially single-chain form, the culture medium (just as the medium used in the subsequent recovery and purification steps) contains a protease inhibitor, such as aprotinin, alpha-1 antitrypsin, alpha-2 macroglobulin, soybean trypsin, etc. Preferably, aprotinin is employed at a concentration of about 5 to 100 KIU/ml, more preferably about 10 KIU/ml in the t-PA production medium.

In a particularly preferred embodiment, the mammalian host cell is a CHO cell, preferably CHO-K1 DUX B11. The necessary nutrients and growth factors for the medium, including their concentrations, for a particular cell line, are determined empirically without undue experimentation as described, for example, in *Mammalian Cell Culture,* Mather, ed. (Plenum Press: NY, 1984) and by Barnes and Sato, *Cell,* 22: 649 (1980). A suitable medium contains a basal medium component such as a DMEM/HAM F-12-based formulation (for composition of DMEM and HAM F12 media and especially serum-free media, see culture media formulations in American Type Culture Collection Catalogue of Cell Lines and Hybridomas, Sixth Edition, 1988, pages 346–349), with modified concentrations of some components such as amino acids, salts, sugar, and vitamins, and optionally containing glycine, hypoxanthine, and thymidine; recombinant human insulin, hydrolyzed peptone, such as PRIMATONE HS™ or PRIMATONE RL™ (Sheffield, England), or the equivalent; a cell protective agent, such as PLURONIC F68™ or the equivalent pluronic polyol; GENTAMYCIN™; and trace elements. The formulations of medium as described in U.S. Pat. No. 5,122,469, characterized by the presence of high levels of certain amino acids, as well as PS-20 as described below, are particularly appropriate.

The glycoproteins of the present invention may be produced by growing cells which express the desired glycoprotein under a variety of cell culture conditions. For instance, cell culture procedures for the large- or small-scale production of glycoproteins are potentially useful within the context of the present invention. Procedures including, but not limited to, a fluidized bed bioreactor, hollow fiber bioreactor, roller bottle culture, or stirred tank bioreactor system may be used, in the later two systems, with or without microcarriers, and operated alternatively in a batch, fed-batch, or continuous mode.

In a preferred embodiment the cell culture of the present invention is performed in a stirred tank bioreactor system and a fed-batch culture procedure is employed. In the preferred fed-batch culture the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed-batch culture can include, for example, a semi-continuous fed-batch culture, wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium Fed-batch culture is distinguished from simple-batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed-batch culture can be further distinguished from perfusion culturing insofar as the supernate is not removed from the culturing vessel during the process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers, etc., and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cells of the culture may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, the present invention contemplates a single-step or multiple-step culture procedure. In a single-step culture the host cells are inoculated into a culture environment and the processes of the instant invention are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture is envisioned. In the multi-stage culture cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

According to a preferred aspect of the invention, fed-batch or continuous cell culture conditions are devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In the growth phase cells are grown under conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (DO_2), and the like, are those used with the particular host and will be apparent to the ordinarily-skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO_2) or a base (e.g., Na_2CO_3 or NaOH). A suitable temperature range for culturing mammalian cells such as CHO cells is between about 30 to 40° C. and preferably about 37° C. and a suitable DO_2 is between 5–90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above the production phase or step may be continuous with the inoculation or growth phase or step.

According to the present invention, the cell-culture environment during the production phase of the cell culture is controlled. In a preferred aspect, the production phase of the cell culture process is preceded by a transition phase of the cell culture in which parameters for the production phase of the cell culture are engaged.

T-PA production in mammalian, e.g., CHO, cells typically employs a semi-continuous process whereby cells are culture in a "seed-train" for various periods of time and are periodically transferred to inoculum fermentors to initiate the cell-amplification process en route to t-PA production at larger scale. Thus, cells used for rt-PA production are in culture for various periods of time up to a maximum predefined cell age. The parameters of the cell culture process, such as seed density, pH, DO_2 and temperature during culture, duration of the production culture, operating conditions of harvest, etc. are a function of the particular cell line and culture medium used, and can be determined empirically, without undue experimentation.

Recovery of the Glycoprotein from the Cell Culture

Following the polypeptide production phase, the glycoprotein of interest is recovered from the culture medium using techniques which are well established in the art. The glycoprotein of interest preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates.

As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The glycoprotein thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, SEPHADEX G-75™; and protein A SEPHAROSE™ columns to remove contaminants such as IgG. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification. One skilled in the art will appreciate that purification methods suitable for the glyco-

protein of interest may require modification to account for changes in the character of the glycoprotein upon expression in recombinant cell culture.

Also of utility within the context of the present invention are purification techniques and processes that select for the carbohydrates of the invention. Such techniques include, for example, ion-exchange soft gel chromatography or HPLC using cation- or anion-exchange resins, wherein the more acidic or more basic fraction is collected, depending on which carbohydrate is being selected for.

In a preferred embodiment, CHO cells capable of producing ht-PA are grown as a suspension in a CHO medium to a predetermined cell density. The cell suspension may be concentrated by cross-flow filtration. Active ht-PA is produced subsequently by the CHO cells suspended in the serum-free expression medium. The ht-PA thus produced is secreted by the CHO cells into the expression medium and may be separated from it by standard techniques.

Several techniques may be used for recovery of the t-PA. For example, at the end of the culture, tangential flow filtration, including high-pressure-tangential flow filtration, can be used to remove the medium containing t-PA from the cells.

The cell culture supernatants may concentrated, diafiltered, and loaded onto an affinity column capable of specific binding of t-PA, typically a lysine affinity column. Under the chromatography conditions employed, t-PA adheres selectively to the affinity column from which it can be recovered and subjected to further purification.

In the diafiltration step, the supernatant of the cell culture on a dialysis membrane may be diafiltered with a dialysis buffer comprising propylene glycol, the solution obtained by diafiltration may be loaded on an affinity column capable of selective binding of t-PA, and t-PA may be eluted from the affinity column with a buffer at a pH of about 5.0 to about 9.0. The affinity column preferably is a lysine affinity column, which is preferably eluted at a pH of about 5.0 to about 8.5, more preferably from about 6.0 to about 8.5. Lysine affinity columns are well known in the art and are commercially available. Suitable columns include Lysine CPG™ (Bioprocessing), ECH Lysine CL™ (Pharmacia), and Lysine Hyper D™ (Biosepra). The gel is preferably equilibrated with a 50 mM Na_2HPO_4 or K_2HPO_4 solution (pH 7.5) prior to loading of the t-PA solution. The elution buffer typically contains 200 mM arginine, and 50 mM Na_2HPO_4 or K_2HPO_4 (pH 7.5). Preferably, the elution buffer additionally contains propylene glycol in a concentration of about 2.5 to about 20%.

After the foregoing recovery/initial purification steps, the feed stream containing 0.5 to 3.0 mg/ml t-PA simultaneously generally contains about 0.05 to 5 ng/ml of DNA as determined by a DNA dot blot assay using ^{32}P-labeled DNA derived from the same cell line, resulting in a calculated clearance of approximately 2×10^4-fold (depending on the source of the lysine resin and on the wash conditions used). To further reduce the level of DNA in the product to less than one picogram per human dose, a specific ion-exchange step may be incorporated into the purification procedure, using commercially available ion-exchange columns, such as a DE-52™ column (Whatman), or DEAE-SEPHAROSE FAST FLOW™ column (Pharmacia).

The purification protocol further includes additional steps that inactivate and/or remove retroviruses that might potentially be present in the cell culture fluid of continuous mammalian cell lines. A significant number of viral clearance steps are available, including additional ultrafiltration/diafiltration steps, treatment with chaotropes such as urea or guanidine, pH extremes, detergents, heat, chemical derivatization, such as formaldehyde, proteases, conventional separation, such as ion-exchange or size exclusion chromatography, organic solvents, etc. The particular step(s) chosen for viral removal is/are not critical aspect(s) of the present invention, and need to meet the following criteria for t-PA: 1. t-PA must be stable under the treatment conditions while the target virus must be sensitive to the treatment, and 2. the "window of clearance" must be maximum. The "window of clearance" is defined for this purpose as the ratio of initial virus titer (spike) in the process fluid prior to the treatment to virus titer after the treatment of the process fluid.

The recombinant human t-PA recovered and purified following the foregoing protocol typically is at least about97–99.9% pure (depending on the lysine resin). If necessary, further purification can be achieved by additional steps, such as cation-exchange chromatography. Accordingly, the product is suitable for therapeutic applications. Various variants of native human t-PA can be purified by essentially the same procedure, and other glycoproteins may be purified by procedures used for their wild-type counterparts, using procedures well-known in the art.

The present invention is further illustrated by the following, non-limiting examples. It is noted that the method of the present invention is equally applicable to the production of other glycoproteins having more than one glycoform in mammalian cell cultures, and the modifications that might become necessary in the course of the adaptation of the exemplified method to the production of different glycoproteins are well within the skill of an ordinary artisan.

EXAMPLE 1

Temperature Shift in the Production of rht-PA

Materials and Methods

CHO cells: The CHO cell line used as the mammalian host cell line was derived from CHO-K1 (ATCC No. CCL61 CHO-K1), and is a CHO-K1 mutant dihydrofolate reductase ($DHFR^-$)-deficient cell line named CHO-K1 DUX-B11 ($DHFR^-$) (obtained from Dr. L. Chasin of Columbia University; Simonsen and Levinson, supra; Urlaub and Chasin, supra).

PS-20 basal medium: The components of this medium are listed in Table 1 below.

TABLE 1

Component	Concentration (mg/l)
Calcium chloride, anhydrous	116.61
Cupric sulfate, pentahydrate	0.0012
Ferric nitrate, nonahydrate	0.05
Ferrous sulfate, heptahydrate	0.417
Potassium chloride	759.0
Magnesium sulfate, anhydrous	48.835
Magnesium chloride, anhydrous	143.05
Sodium phosphate, monobasic, monohydrate	62.5
Sodium phosphate, dibasic, anhydrous	71.02
Zinc sulfate, heptahydrate	0.4315
Linoleic acid	0.294
Lipoic acid (DL thioctic acid)	0.735
Putrescine, dihydrochloride	0.5635
Sodium pyruvate	385.0
Alanine	31.15
Arginine, monohydrochloride	780.5
Asparagine, monohydrate	52.53
Aspartic acid	46.55
Cysteine, monohydrochloride, monohydrate	122.92
Cystine, dihydrochloride	31.285

TABLE 1-continued

Component	Concentration (mg/l)
Glutamic acid	51.45
Glutamine	1606.0
Histidine, monohydrochloride, monohydrate	94.36
Isoleucine	66.29
Leucine	98.35
Lysine, monohydrochloride	200.75
Methionine	30.68
Phenylalanine	50.36
Proline	120.75
Serine	57.75
Threonine	89.15
Tryptophan	15.14
Tyrosine, disodium salt, dihydrate	79.125
Valine	87.95
Biotin	0.0256
D-Calcium pantothenate	3.68
Choline chloride	50.86
Cyanocobalamin (B12)	4.76
Folic acid	6.55
i-Inositol	66.60
Nicotinamide	2.1295
Pyridoxal, monohydrochloride	2.000
Pyridoxine, monohydrochloride	0.217
Riboflavin	0.3330
Thiamine, monohydrochloride	3.190
Glucose	4301.0
Sodium bicarbonate	2440.0
Sodium chloride	5900.0
Pluronic F-68 Prill	1000.0
HEPES	2383.0
Phenol Red	8.10

For convenience, the solid ingredients of the medium may be combined together with the amino acids, and this mixture may be stored as a single unit.

Type I/Type II t-PA assay:

1. Thaw cell culture supernatant sample (If whole broth, spin out cells in centrifuge.)
2. Add 2 μl freshly thawed plasminogen to 400 μl of sample.
3. Incubate at 37° C. for 60 minutes.
4. Add 20 μl freshly-thawed 1 M DTT and 400 μl 8 M guanidine-HCl/50 mM TRIS/3.2 mM EDTA solution.
5. Incubate at 37° C. for 15 minutes.
6. Transfer to vial and load 250 μl for assay on HP 1090™ HPLC using the following conditions:
   ZORBAX™ C8 column at 40° C.;
   Monitoring of eluents by fluorescence (excitation at 275 nm, emission at 340 nm);
   Running each sample with the following 70-minute method where eluent A is 0.1% trifluoroacetic acid (TFA) in water and eluent B is 0.1% TFA in acetonitrile:
   0 to 5 min.—75% A (and 25% B)
   5 to 35 min.—a linear gradient from 75% A to 60% A
   35.1 to 45 min.—0% A
   45 min. to 70 min.—75% A to re-equilibrate the column
   Type I/Type II fragments elute after approximately 25 minutes, and peak areas are integrated to determine relative quantities.

Protocol

Recombinant ht-PA-producing CHO cells were carried in spinner flasks passaged every 3 or 4 days (at a density of 0.1% packed cell volume (PCV)) in selective medium (PS-20 basal medium supplemented with 500 mM methotrexate, 10 mg/L recombinant human insulin (rh-insulin), 0.1 ml/L trace elements, and 0.05 ml/L lipid-ethanol). Sufficient culture was removed to seed 15 ml of medium at 0.2% PCV, and placed in a 50 ml sterile Falcon tube. The culture was centrifuged for 10 minutes at 700–1000 rpm and the supernatant was poured off. Upon addition of 15 ml of fresh selective medium, the culture was agitated gently to resuspend cells. Five ml of culture was placed in three T-25 flasks (25 cm^2 T-flasks). The caps were left loosened to allow equilibration with incubator atmosphere and the flasks were placed in a 33° C. or 31° C. incubator with 5% carbon dioxide. After 5 to 8 days of incubation, the cells were counted using a hemacytometer and/or by checking packed cell volume, and viability was checked using trypan blue. The culture was removed from the flasks, centrifuged for 10 minutes at 2000–2200 rpm, and the supernatant was assayed for rht-PA glycosylation. Alternatively to the T-25 flasks, the cells were cultured using 60-mm cell culture plates in triplicates.

Supernatants were frozen at −20° C. or −70° C. until the Type I/II t-PA analysis took place.

For spinner experiments, the foregoing protocol was used, except the cells were passaged into 200 ml of fresh medium (with initial PCV of 0.1%), in a 250-ml spinner flask. The caps were closed tightly on the flask, which was then placed in the 33° C. or 31° C. incubator on a magnetic stir plate at 60 rpm.

The mini-fermentor experiments were performed under standard t-PA production conditions in fed-batch mode as noted herein in 5-liter stirred tank bioreactors (Applikon, Foster City, Calif.). The temperature was shifted to 33° C. or 31° C. on day 2 of the production phase.

For control experiments, the foregoing experiments were followed, except that incubation took place at 37° C. Further, experiments were performed as above in 12-K fermentors over the course of 200 hours and the percentage of Type I t-PA was assessed.

For the experiments herein and below, culture conditions were usually changed on day 1 (e.g., addition of different media components) and day 2 (e.g., temperature shift).

Results

t-PA site occupancy at Asn-184 was found to be relatively consistent across a variety of scales (T-flask, spinner, and 80-L, 400-L, 2000-L, and 12,000-L fermentors) (FIG. **2**) and from run to run in production. The factors that can affect site occupancy include those factors affecting oligosaccharide-dolichol availability (dolichol phosphate, lipids, and hormones), factors affecting protein translation elongation rate (e.g., cyclohexamide), factors affecting oligosaccharyltransferase activity or protein folding rate (e.g., dithiothreitol), and factors acting through unknown mechanisms, such as time in culture. Illustrating the lattermost phenomenon, FIG. **3** shows graphs of the percentage of Type I human t-PA in cell cultures of CHO cells at 37° C. as a function of 12K-fermentation run time, with each graph line representing a different run. These results show that site-occupancy increases over the course of a batch culture (over culture length).

FIG. **4** shows that reducing temperature increases t-PA site occupancy. Specifically, FIG. **4**A shows the results of laboratory-scale experiments performed for 5–7 days (T-flasks and spinner flasks). In the control experiments, where in the production phase the temperature remained at 37° C., the product contained about 38% Type I t-PA. In contrast, in the experiments where in the production phase the temperature was lowered to 33° C., the t-PA product obtained contained about 43–46% Type I t-PA. FIG. **4**B

shows the results of bioreactor experiments, indicating that the lower temperature similarly yields a higher percentage of Type I t-PA.

EXAMPLE 2

Butyrate Addition in the Production of rht-PA

Materials and Methods

The quantity of cells needed for a 0.2% packed cell volume seed density was centrifuged at approximately 700×g for 10 minutes, and resuspended in 25 cm^2 T-flasks in the appropriate fresh medium for each test case. The T-flasks were set up in triplicate with 5 mL in each flask and incubated for 3 to 4 days at 37° C. and 95% air 5% CO_2. Sodium butyrate was added to a concentration of 0.375 mM, 0.75 mM, or 1.5 mM at the time of inoculation for the 25 cm^2 T-flasks. Cells were also cultured in spinner flasks at volumes of 100 ml with similar butyrate concentrations to the T-flask cases. For fermentor experiments, butyrate was added on the second day of the experiments. T-flask cultures were also carried out at 33° C. and 37° C. with no butyrate additions. The data shown for the T-flask cultures at 33° C. are from two triplicate experiments (n=6). T-PA site-occupancy was analyzed using the method of Example 1.

Results

FIG. **5** shows that the presence of sodium butyrate at concentrations of 0.375 to 1.5 mM at the time of inoculation increased t-PA site-occupancy in the T-flasks at 37° C. The same increased effect was observed for the spinner flask and fermentor experiments.

FIG. **6** shows that for the 60-mm culture plate experiments, temperature shifts to 33° C. and 31° C. had the largest effect and increased t-PA site-occupancy gradually up to 6%. 0.75 mM butyrate increased the Type I content slightly (about 1%) compared to no butyrate (FIG. **6**). In contrast, a further increase of the butyrate concentration (1.5 mM) lowered site-occupancy at 37° C. and 33° C., but increased it at 31° C. (FIG. **6**).

FIG. **7** shows the effect of temperature and butyrate on t-PA site-occupancy in 5-liter bioreactors. The Type I content was analyzed on days 5–7. Decreasing temperature from 37° C. to 33° C. increased t-PA site occupancy. However, increasing the butyrate concentration from 0.75 to 1.5 mM decreased the Type I content at both temperatures. This confirms the data obtained in 60-mm plate experiments (see FIG. **6**).

EXAMPLE3

Cell-cycle Inhibitor Addition in the Production of rht-PA

Introduction

Temperature reduction, culture length, and butyrate addition were found to increase the glycosylation of t-PA at the Asn-184 site, as noted in Examples 1 and 2. All of these factors correspond with decreased cell growth rate, leading to the hypothesis that glycosylation and site-occupancy are cell-cycle dependent. This hypothesis was tested by performing two additional experiments reflected in this example using cell-cycle inhibitors (quinidine and thymidine).

Materials and Methods

Cells were cultured as for the sodium butyrate experiment described in Example 2. Thymidine was dissolved in water and sterile filtered for a 33–36× stock solution. Quinidine was made up in dimethyl sulfoxide (DMSO) and filtered to make a 1000–1800× stock. Thymidine and quinidine were added to the cultures at the time of inoculation to final concentrations of 250 μg/mL and 90 μM, respectively. T-PA site-occupancy was analyzed using the method of Example 1.

Results

The cell cycle analysis for the control, the quinidine, and the thymidine is shown in Table 2, as determined by model F_AN1_T3.MOD.

TABLE 2

Analysis	Cell-Cycle Analysis Cell Cycle Control	Quinidine	Thymidine
G0/G1	53.91% at 84.70	67.34% at 84.65	32.58% at 84.60
G2-M	11.72% at 165.58	8.55% at 165.45	10.09% at 165.36
S	34.37%	24.11%	57.33%
G2/G1	1.95	1.95	1.95
% CV	1.97	1.79	1.99

FIG. **8** shows that site occupancy and cell-cycle position vary similarly over time in a culture. FIG. **9** shows that quinidine blocks the cells in the G1/G1 phase (with 67% in the G0/G1 phase) and results in increased site occupancy as compared to the control with no cell-cycle inhibitor (54% in the G1/G1 phase), and thymidine causes the cells to accumulate in the S/G2 phases (with 33% in the G0/G1 phase) and results in decreased site occupancy as compared to the control. These results confirmed that factors that increase the proportion of cells in the G0/G1 phase increase site occupancy.

EXAMPLE 4

Divalent Metal Cation, Nucleotide Sugar Precursor, and Hormone Addition in the Production of rht-PA

Materials and Methods

All experiments were done in 60-mm culture dishes using the procedure described in Example 1 except that a Mn salt, Fe salt, nucleotide sugar precursor, or hormone was added to the growth medium during the growth phase, with Mn or Fe salt or nucleotide sugar precursor added on day 1. All plates were inoculated at a seeding density of 0.1% PCV and a working volume of 6–8 ml. All cases were done in triplicates. Plates were incubated at 37° C. in CO_2 incubators.

The amounts of $MnCl_2$ or ferric citrate in the growth medium during the growth phase were increased. For $MnCl_2$ 3 nM was the control, with increasing amounts of $MnCl_2$ at concentrations of 10 nM, 100 nM, 1 μM, 10 μM, and 100 μM. For ferric citrate, the control was no salt, with increasing amounts at 10, 50, and 100 μM. The amounts of uridine, adenosine, and guanosine were 0.5 mM each and the amount of mannose was 5 g/l (guanosine and mannose were combined), and -GHT is selective medium minus glycine, hypoxanthine and thymidine.

The amounts of tri-iodothyronine and thyroxine employed in the culture medium were increased relative to a control with no hormone, with amounts of 1 nM, 10 nM, or 100 nM tri-iodothyronine, or 1 nM, 10 nM, or 100 nM thyroxine.

T-PA site-occupancy was analyzed on days 4–6 (manganese, iron, or nucleotide sugar precursor experiment) or on day 7 (hormones) using the method described in Example 1.

Results

The effect of increasing manganese concentration on t-PA site-occupancy (triplicate runs) is shown in FIGS. **10**A and **10**B. Supplementing the medium with additional manganese over the 3 nM control value significantly increased the t-PA

Type I content (improved site-occupancy) about 2.5%. A positive titration effect was observed between 3 nM and 100 nM. No further improvement occurred when increasing the concentration up to 100 μM, which is still an effective concentration.

Oligosaccharyltransferase requires Mn^{2+} ions for maximal activity, but other divalent metal cations with an octahedral coordination geometry, including Mg, Ca, and Fe, will support transfer, albeit at reduced rates (Hendrickson and Imperiali, suprs, and Kaufman et al., supra). Hence, the effect of Fe^{2+} on t-PA site-occupancy was investigated. As is evident from FIG. **11**, the addition of 10–100 μM Fe^{2+} (ferric citrate) increased t-PA site occupancy gradually up to about 4%.

Increasing the availability of nucleotide sugar precursors (e.g., nucleosides, mannose, glycine, thymidine, or hypoxanthine) did not improve site-occupancy. Moreover, the addition of uridine and guanosine decreased t-PA site-occupancy about 2% (see FIG. **12**).

The effect of thyroid hormones (thyroxine and tri-iodothyronine) on t-PA site-occupancy is shown in FIGS. **13**A and **13**B. These hormones increased site-occupancy about 2%, and it is expected that other plasma components as defined above would similarly have such an effect.

In conclusion, several culture conditions that affect t-PA N-glycosylation site-occupancy have been identified. These factors are potentially useful to further improve product consistency. T-PA site occupancy at Asn-184 is relatively consistent across a variety of conditions, including a variety of scales (T-flask, spinner, 80-liter, 400-liter, 2000-liter, and 12,000-liter), and from run to run in production. Factors that increase the proportion of cells in the G0/G1 phase, such as temperature, butyrate, and cell-cycle inhibitors, increase site occupancy, as do increased amounts of certain divalent metal cations and/or plasma components preferably present during the whole cultivation time.

The entire disclosures of all citations cited throughout the specification, and the references cited therein, are hereby expressly incorporated by reference.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those ordinarily skilled in the art that various modifications may be made without diverting from the overall concept of the invention. All such modifications are intended to be within the scope of the present invention.

What is claimed is:

1. A process for producing a human glycoprotein having multiple glycoforms, comprising culturing Chinese hamster ovary cells expressing nucleic acid encoding said glycoprotein in a serum-free medium in a production phase at a temperature of about 30° C. to 35° C. and in the presence of about 0 to 2 mM of a butyrate salt, wherein the process produces an increased percentage of glycoprotein molecules having one glycoform relative to an identical process performed at 37° C. in the absence of butyrate.

* * * * *

EXHIBIT Y

US006716602B2

(12) **United States Patent**
Andersen et al.

(10) **Patent No.: US 6,716,602 B2**
(45) **Date of Patent: Apr. 6, 2004**

(54) **METABOLIC RATE SHIFTS IN FERMENTATIONS EXPRESSING RECOMBINANT PROTEINS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **10/000,655**

(22) Filed: **Nov. 1, 2001**

(65) **Prior Publication Data**

US 2002/0164700 A1 Nov. 7, 2002

Related U.S. Application Data

(60) Provisional application No. 60/245,962, filed on Nov. 3, 2000.

(51) **Int. Cl.**[7] **C12P 21/06**

(52) **U.S. Cl.** **435/69.1**; 435/69.4; 435/71.2; 435/252.8; 435/243

(58) **Field of Search** 435/69.1, 69.4, 435/71.2, 252.8, 243

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,565,785 A	1/1986	Gilbert et al.	435/317
4,673,641 A	6/1987	George et al.	435/68
4,710,473 A	12/1987	Morris	435/320
4,738,921 A	4/1988	Belagaje et al.	435/68
4,795,706 A	1/1989	Hsiung et al.	435/172.3
5,342,763 A	8/1994	Schwartz	435/69.1
5,612,198 A *	3/1997	Brierley et al.	
5,639,635 A	6/1997	Joly et al.	435/69.7
5,789,199 A *	8/1998	Joly et al.	435/69.1
6,410,270 B1	6/2002	Strittmatter et al.	435/696

FOREIGN PATENT DOCUMENTS

DE 19943919 3/2001

OTHER PUBLICATIONS

Flamez et al., Production in *Escherichia coli* of a functional murine and murine::human chimeric F (ab')2 fragment and mature antibody directed against human placental alkaline phosphatase, 1995, Journal of Biotechnology, vol. 42, pp. 133–143.*

Knorre et al., High cell density fermentation of recombinant *Escherichia coli* with computer–controlled optimal growth rate, 1991, Ann. NY. Acad. Sci., vol. 646, pp. 300–306.*

Skerra et al., Use of tetracycline promoter for the tightly regulated production of a murine antibody fragment in *Escherichia coli*, 1994, Gene, vol. 151, pp. 131–135.*

Gram et al., A novel approach for high level production of a recombinant human parathyroid hormone fragment in *Escherichia coli*, 1994, Bio/Technology, vol. 12, pp. 1017–1023.*

Cheng et al., "A Novel Feeding Strategy for Enhanced Plasmid Stability and Protein Production in Recombinant Yeast Fedbatch Fermentation." *Biotechnol. Bioeng.* 56:23–31 (1997).

Cruz et al., "Metabolic Shifts by Nutrient Manipulation in Continuous Cultures of BHK Cells." *Biotechnol. Bioeng.* 66:104–113 (1999).

Cruz et al., "Metabolically Optimised BHK Cell Fed–Batch Cultures." *J. Biotechnology.* 80:109–118 (2000).

Curless et al., Biotechnol. Prog. 1990, 6:149.

Ryan et al., Biotechnol. Prog. 1996, 12:596.

Yoon et al., Biotechnol. Prog. 1994, 43:995.

Villa–Komaroff, et al., Proc. Natl. Acad. Sci. USA 1978, 75:3727–373.

DeBoer et al., Proc. Natl. Acad. Sci. USA 1983, 80:21–25.

Sheibani, Prep. Biochem. Biotechnol. 1999, 29:77.

Gossen et al., Curr. Opin. Biotechnol. 1994, 5:516.

De Vos et al., Curr. Opin. Biotechnol. 1997, 8:547.

Chevalet et al., Biotechnol. Bioeng. 2000, 69:351.

Schroeckh et al., J. Biotechnol. 1999, 75:241.

Staijen et al., J. Bacteriol. 1999, 181:1610.

Newman and Fuqua, Gene 1999, 277:197.

Liu et al., Biotechniques 1998, 24:624.

Gallia and Khalili, Ongogene 1998, 16:1879.

Haldimann et al., J. Bacteriol. 1997, 179:5903.

Treuner–Lange et al., J. Bacteriol. 1997, 179:4501.

San et al., Ann. NY Acad. Sci. 1994, 721:268.

Bishai et al., J. Bacteriol. 1994, 176:2914.

Lama and Carrasco, Biochem. Biophys. Res. Commun. 1992, 188:972.

Nielson et al., Mol. Microbiol. 1991, 5:1961.

Alksne and Rasmussen, J. Bacteriol. 1997, 179:2006.

Everett et al., Microbiology 1995, 141:419.

(List continued on next page.)

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(57) **ABSTRACT**

The invention provides a method for increasing product yield of a polypeptide of interest produced by recombinant host cells, where expression of the polypeptide by the recombinant host cells is regulated by an inducible system. More specifically, the method involves culturing the recombinant host cells under conditions of high metabolic and growth rate and then reducing the metabolic rate of the recombinant host cells at the time of induction of polypeptide expression. In particular, the invention provides a method of increasing product yield of an antibody, growth factor, or protease produced by a recombinant *E. coli* host cell regulated by an inducible system.

39 Claims, 4 Drawing Sheets

OTHER PUBLICATIONS

Curless et al., "Phosphate Glass as a Phosphate Source in High Cell Density *Escherichia coli* Fermentations" *Biotechnol. Prog.* 12:22–25 (1996).

Hellmuth et al, "Effects of growth rate on stablility and gene expression of recombinant plasmids during continuous and high cell density cultivation of *Escherichia coli* TG1" *Journal of Biotechnology* 32:289–298 (1994).

Lee and Chang,, "High Cell Density Culture of a Recombinant *Escherichia coli* Producing Penicillin Acylase in a Membrane Cell Recycle Fermentor" *Biotech and Bioengin* 36:330–337 (1990).

Lee, Sang Yup, "High cell–density culture of *Escherichia coli*" *Tibtech* 14:98–105 (Mar. 1996).

Vallejo et al., "Renaturation and purification of bone morphogenetic protein–2–produced as inclusion bodies in high–cell density cultures of recombinant *Escherichia coli* " 94:185–194 (2002).

Wilms et al., "High–Cell Density Fermentation for Production of L–N–Carbamoylase Using an Expression System Based on the *Escherichia coli* rhaBAD Promoter" *Biotech and Bioengin* 73:95–103 (2001).

* cited by examiner

FIG. 1

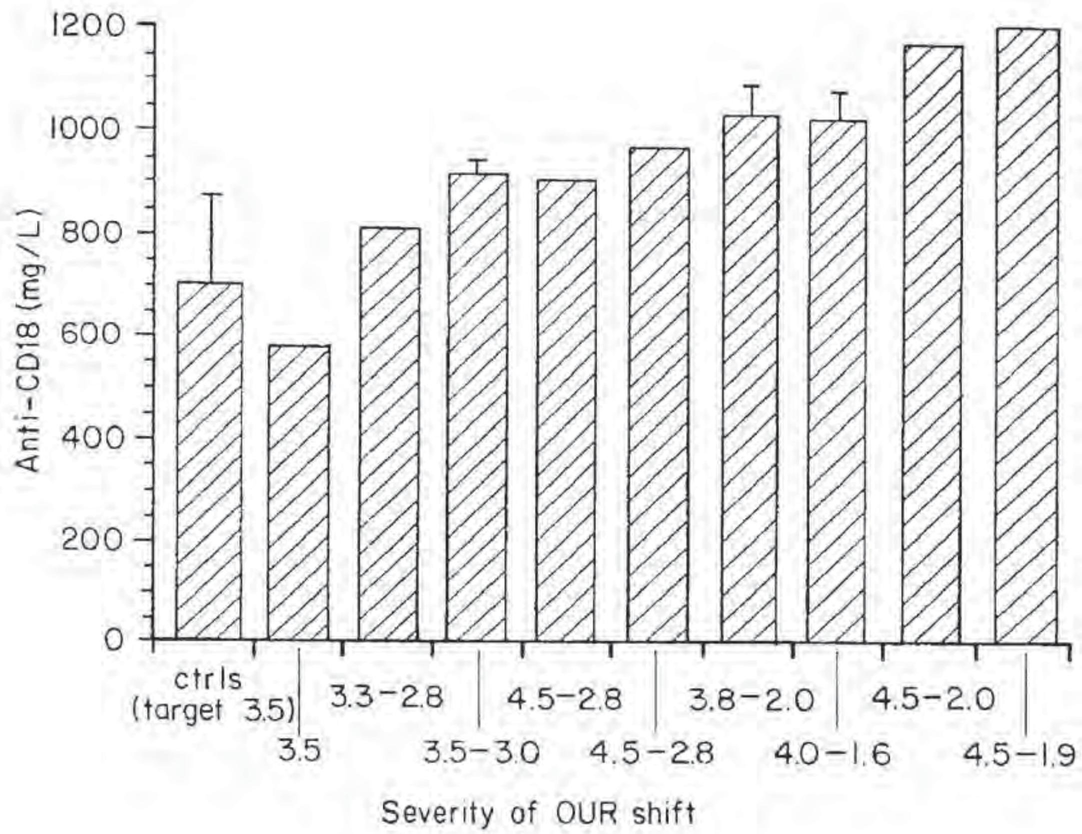


FIG. 2

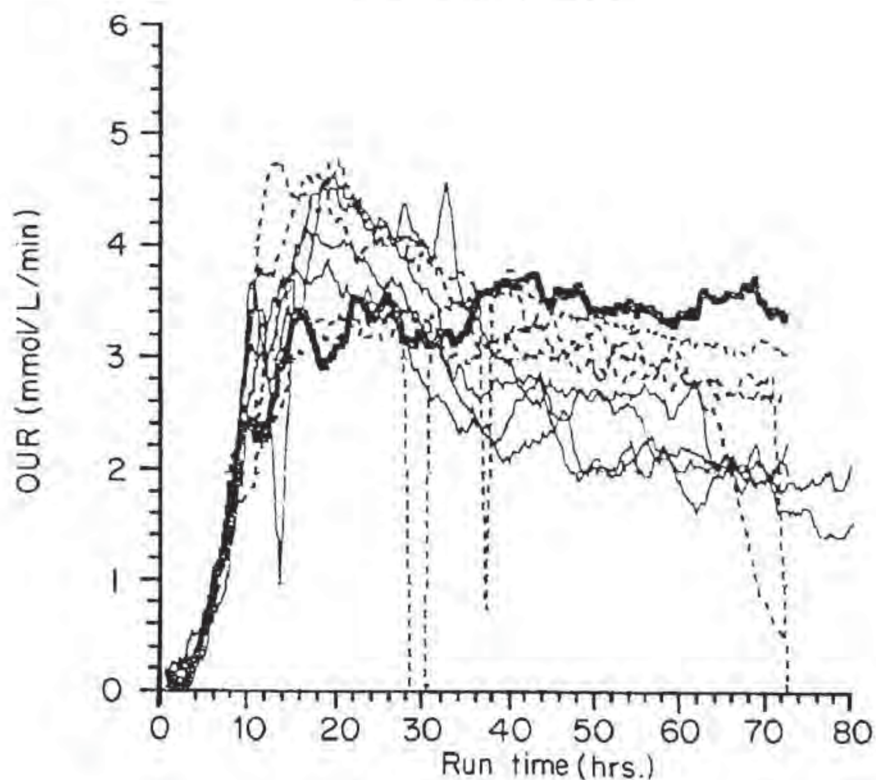


FIG. 3

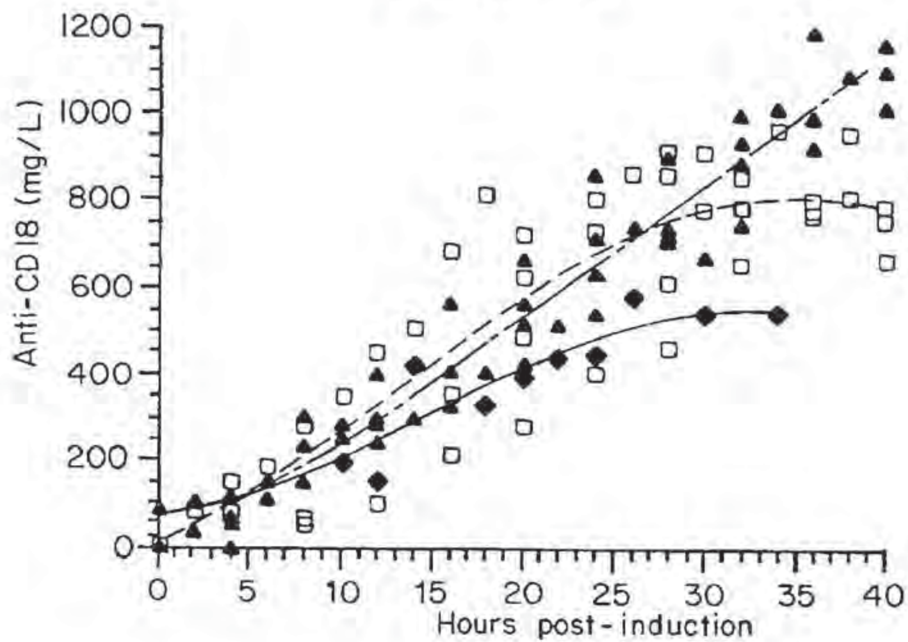


FIG. 4A

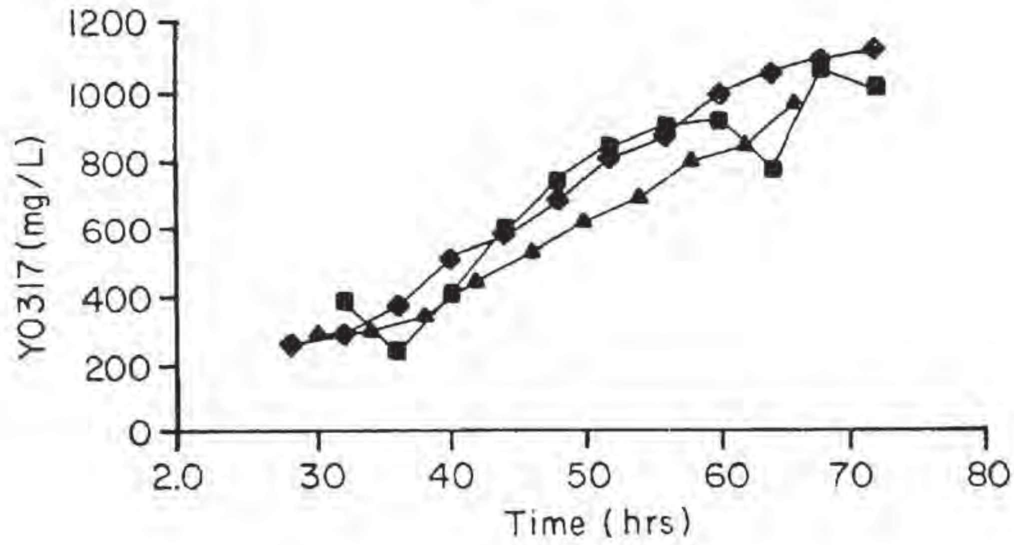


FIG. 4B

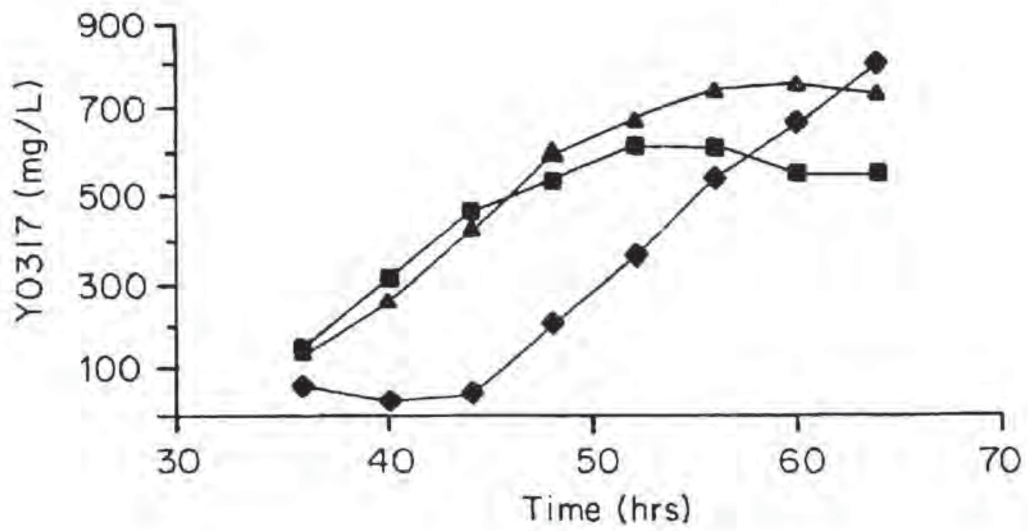


FIG. 5A

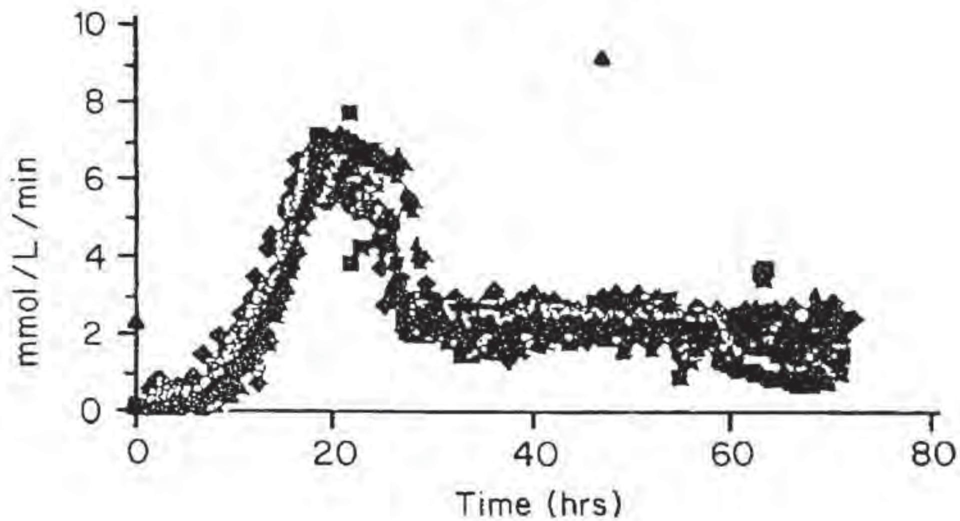
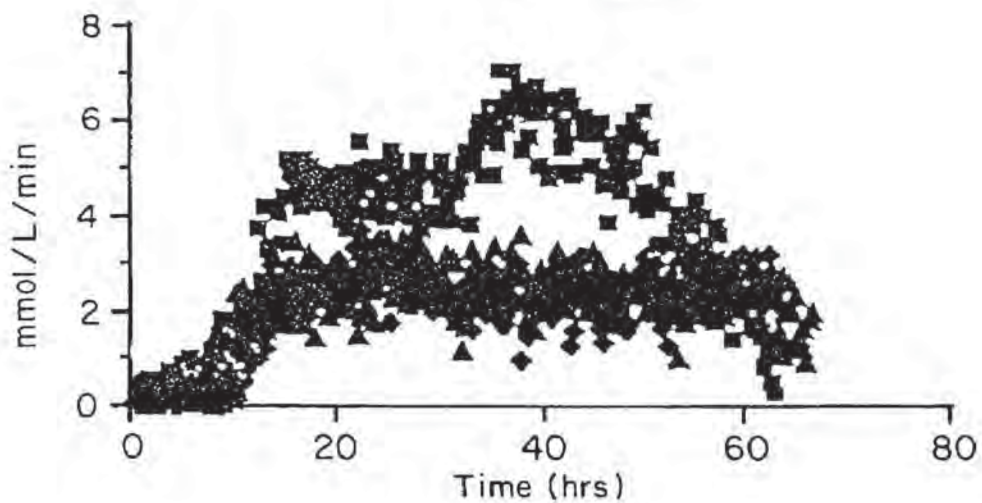


FIG. 5B



METABOLIC RATE SHIFTS IN FERMENTATIONS EXPRESSING RECOMBINANT PROTEINS

This application claims priority under 35 U.S.C. §119(e) from U.S. Provisional Application Serial No. 60/245,962, filed Nov. 3, 2000.

FIELD OF THE INVENTION

The present invention relates to improvements in product yield from fermentation to produce recombinant proteins, particularly in prokaryotic and simple eukaryotic systems. More particularly, this invention greatly increases the yield of properly assembled proteins in large scale fermentations.

BACKGROUND OF THE INVENTION

The production of large quantities of relatively pure, biologically active polypeptides and proteins is important economically for the manufacture of human and animal pharmaceutical formulations, enzymes, and other speciality chemicals. Recombinant DNA techniques have become the method of choice to produce large quantities of exogenous proteins using bacteria and other host cells. The expression of proteins by recombinant DNA techniques for the production of cells or cell parts that function as biocatalysts is also an important application.

Producing recombinant protein involves transfecting host cells with DNA encoding the protein and growing the cells under conditions favoring expression of the recombinant protein. The prokaryote *E. coli* is favored as host because it can be made to produce recombinant proteins in high yields. Numerous U.S. patents on general bacterial expression of DNA encoding proteins exist, including U.S. Pat. No. 4,565,785 on a recombinant DNA molecule comprising a bacterial gene for an extracellular or periplasmic carrier protein and a non-bacterial gene; U.S. Pat. No. 4,673,641 on co-production of a foreign polypeptide with an aggregate-forming polypeptide; U.S. Pat. No. 4,738,921 on an expression vector with a trp promoter/operator and trp LE fusion with a polypeptide such as IGF-I; U.S. Pat. No. 4,795,706 on expression control sequences to include with a foreign protein; U.S. Pat. No. 4,710,473 on specific circular DNA plasmids such as those encoding IGF-I; U.S. Pat. No. 5,342,763 on improving expression in bacteria by manipulating oxygen delivery; and U.S. Pat. No. 5,639,635 on secretion of the expressed protein into the bacterial periplasm.

Recombinant proteins become less expensive if the fermentation yield improves. Yield depends upon the rate at which the recombinant protein is properly folded and assembled protein is formed and upon the length of time over which the protein is produced.

The recombinant protein expression rate is typically affected by the growth and metabolic rates of the cells. At higher growth rates, the rate at which a protein can be expressed when induced typically increases (Curless et al., Biotechnol. Prog. 1990, 6:149). However, upon induction, high protein expression rates may not always lead to high rates of formation of active, properly formed products. In other words, while the quantity of protein translated may be maximized, other factors may compromise the quality of the product, such as degradation of the protein by proteases or other detrimental post-translational modifications (Ryan et al., Biotechnol. Prog. 1996, 12:596; Yoon et al., Biotechnol. Prog. 1994, 43:995). Efficient fermentation requires balancing growth rate against yield of usable protein; compromises

between these factors result in a decrease of the overall yield below its theoretical potential. Consequently, some intermediate growth rates may be more favorable for the production of high quantities of high quality product.

An added complication is that induction of recombinant protein expression essentially highjacks the cellular protein assembly process to make large quantities of a product with no benefit, and often with significant detriment, to the cell. In fact, for cases in which induction is triggered by phosphate depletion using the alkaline phosphatase promoter, growth rate is dramatically curtailed by the phosphate starvation itself. This effect does not affect the metabolic rate, however.

Thus, there is a need in the art to increase the yield of usable recombinant protein production. The present invention advantageously and unexpectedly addresses this need by permitting high levels of protein synthesis, assembly and folding. Because different factors may play critical roles in maximizing usable protein yield prior to induction during the growth phase of the culture, and post-induction, the independent control of these two factors can lead to improved yields of usable products, such as for the case of soluble, properly folded and assembled antibody fragments.

SUMMARY OF THE INVENTION

The invention provides a method for increasing product yield of a polypeptide of interest produced by recombinant host cells, where expression of the polypeptide by the recombinant host cells is regulated by an inducible system. More specifically, the method involves culturing the recombinant host cells under conditions of high metabolic and growth rate, then reducing the metabolic rate of the recombinant host cells at the time of induction of polypeptide expression.

In a specific embodiment the invention provides a method of increasing product yield of an antibody, growth factor, or protease produced by a recombinant *E. coli* host cell regulated by an inducible system.

In a further specific embodiment, the invention provides a method of increasing the yield of actively folded proteins having different structures, for example Fab'$_2$ versus Fab Fv antibody fragments, by selecting the time to initiate reduction in metabolic rate (the rate shift), the rate of adjustment (shift) of the metabolic rate, and the final metabolic rate. Adjusting these parameters of the invention enhances the yield of correctly folded proteins having different secondary and tertiary structures, interaction and refolding characteristics, size and contact area, and other factors that can affect protein assembly and function.

DESCRIPTION OF THE DRAWINGS

FIG. **1** shows anti-CD18 yield (titer) in a series of anti-CD18 Fab'$_2$ fermentations. X-axis: approximate oxygen uptake rate which reflects the severity of the oxygen use rate shift.

FIG. **2** shows actual oxygen uptake rate profile from the fermentations in FIG. **1**. Titers exceeding 1000 mg/L are represented by a thin solid line (——); titers between 800–900 mg/L are represented by a dotted line (-----); and the unshifted control (~600 mg/L) is represented by a heavy solid line (——).

FIG. **3** shows the titer results for quantitating antibody production of the various runs as a function of fermentation time. A less severe shift is represented by squares (---□---), a more severe shift is represented by triangles (▲---) and the control is represented by diamonds (♦).

FIGS. 4A and 4B show titer profiles for a series of anti-VEGF fermentations or without (B) oxygen use rate (OUR) shifts. The graphs each present data from three runs. Run 1 is represented by diamonds (♦), Run 2 is represented by squares (■) and Run 3 is represented by triangles (▲).

FIGS. 5A and 5B show OUR profiles for the runs in FIG. 4. Run 1 is represented by diamonds (♦), Run 2 is represented by squares (■) and Run 3 is represented by triangles (▲).

DETAILED DESCRIPTION

The present invention advantageously provides a method for increasing yield of a heterologous recombinant protein produced by recombinant host cells by first increasing the protein production capacity of the cells in culture by culturing the cells at a high growth rate, and then decreasing metabolic rate of the cells (rate shift) to permit proper folding or assembly of the heterologous protein. In a specific embodiment, implementing a high growth rate was found to extend the period of heterologous gene expression. In a further specific embodiment, the metabolic rate shift increases the yield of properly folded and, if appropriate, assembled protein. These features together increase fermentation efficiency.

The invention is based on observations in a number of *E. coli* fermentations producing anti-CD18 Fab'$_2$ and anti-VEGF Fab, that the deliberate down shifting of the cellular metabolic rate of the cells (by manipulating the oxygen transfer rate and correspondingly, the glucose feed rate in the fermentor) significantly improves product yields. In particular, growing the cells at a relatively high metabolic rate, and then dramatically shifting down the metabolic rate after the induction of antibody expression greatly improves yield. A substantial amount of data demonstrates that this approach extends the period of antibody fragment assembly, leading to significantly higher titers.

These experiments also established that for any given heterologous protein expression system, i.e., the nature of the protein and characteristic of the host, tuning the metabolic rate shift further increases useful protein yields. The tuning variables include the tuning of the metabolic rate shift, the step-down rate (rate of decrease in available oxygen or carbon/energy source, or both), and the final metabolic rate (available oxygen level, available carbon/energy source level or both).

Consequently, post-induction, the protein expression rate can be controlled by manipulating the metabolic rate, one common measure of which is the oxygen uptake rate of the cells in the fermentor. Metabolic rate control can be achieved by controlling the feeding of the primary carbon source, commonly glucose, often in conjunction with manipulation of fermentor parameters such as agitation rate and back pressure, to control the oxygen transfer rate to the cells. Conversely, metabolic rate control can be achieved by limiting the available oxygen, in conjunction with a reduction in the glucose feed rate. Similar trade-offs exist between protein synthesis rate and the rate of formation of usable product for controlling the metabolic rate post-induction as previously discussed for controlling growth rate pre-induction. For the case of maximizing the yield of antibody fragments, the rate and period of assembly of soluble, active product from the individual light-chain and heavy-chain polypeptides occurs at some favorable post-induction metabolic rate.

While data in the literature suggests that fermentations may have a favorable growth rate for protein production, the

results in this application show that the shifting of metabolic rates in different phases of the fermentation provides a critical benefit. In other words, we see significantly improved product yields by shifting the metabolic rate compared to the titers obtained by running the fermentation at a previously favorable, constant metabolic rate. While all of the data to date has been obtained using fermentations producing antibody fragments in *E. coli*, this approach applies to a variety of proteins, including growth hormone, expressed in other prokaryotic and simple eukaryotic systems.

As used herein, "reducing metabolic rate" or "shifting down metabolic rate" means altering the host cell culture conditions such that the host cells undergoing rapid growth and expansion reduce (or stop) growth and expansion. For the case of cells already in a reduced growth state, the rates of oxygen uptake and the corresponding rates of uptake of a carbon/energy source are reduced. Since, in the case of respiring cells, the metabolic rates are determined primarily by the rate at which the cell oxidizes the available carbon/energy source using the available oxygen, the metabolic rate can be reduced by limiting either of these two reactants. So reduction of metabolic rate can result from inter alia (1) reducing the amount of available oxygen in the cell culture (i.e., fermentation); (2) reducing the amount of available carbon/energy sources; or (3) reducing both.

The term "available oxygen" refers to oxygen that can be used by the cells. "Decreasing available oxygen" can be effected by decreasing the oxygen transfer rate to the culture, or decreasing the oxygen transfer by the cells or both. Often it is desirable to reduce the feed rate of glucose (or alternative carbon/energy source) correspondingly, and so the dissolved oxygen concentration may be decreased or not, depending on which reactant most directly limits respiration.

As used herein, the phrase "carbon/energy source" refers to a source of carbon and energy for the cells. Examples of such a source include glycerol, succinate, lactate, and sugars such as, e.g., glucose, lactose, sucrose, and fructose. The selection of the particular carbon/energy source to employ will depend mainly on the characteristics of the host cell. The preferred carbon/energy source for *E. coli* fermentation is glucose.

Thus, decreasing available carbon/energy sources can mean reducing the concentration or feed rate of the carbon/energy source, or reducing the rate of transfer to the host cells or uptake by the host cells of the carbon/energy source, or both.

As used herein "culturing the host cells under conditions of high metabolic and growth rate" means establishing the host cell culture conditions to favor growth. e.g., by providing unrestricted or relatively high feed rates of nutrients energy and oxygen, such that the cells have rapid growth and metabolic rates prior to reducing metabolic rate to increase "product yield". Under these conditions host cell doubling time decreases towards its minimum and host cell metabolism increases exponentially towards its maximum, potentially achieving either or both conditions. Measurement of metabolic And growth rates is easily determined using routine techniques, including but not limited to measurement of increases in cell number, measurement of increases in cell density (e.g., optical density), measurement of pH changes of the growth medium containing the cell, measurement of accumulated metabolites, measurement of heat production, measurement of electrical conductivity of the medium, measurement of nutrient feed rates, and measurement of oxygen uptake and carbon dioxide evolution rates.

As used herein, the term "product yield" refers to the quantity of useful recombinant protein produced by a fermentation system. Protein quantity is readily determined using routine techniques, including but not limited to chromatography, spectrometry gel electrophoresis, immunoassay, coomassie blue or silver staining, and the Lorry essay. Protein quality is further evaluated by comparing product to a standard in appropriate biophysical or activity assays, e.g., high performance liquid chromatography, spectroscopic analysis, or immunoassay. Activity assays can reveal properly folded or assembled functional protein. Thus, properly assembled antibody may bind antigen, preferably with similar affinity as a control antibody. A properly assembly growth factor, hormone, or cytokine will bind its cognate receptor and induce cell signaling, again in a manner comparable to that of wild-type growth factor, hormone, or cytokine. A properly refolded protease will cleave peptide bonds with similar specificity to that of a wild-type protease.

As used herein, "polypeptide of interest" refers generally to peptides and proteins having more than about 10 amino acids. The polypeptides may be endogenous to the bacterial host cell, or, preferably, may be exogenous to the host cell, such as yeast polypeptides, or more preferably, mammalian polypeptides. Examples of bacterial polypeptides include, e.g, alkaline phosphatase and beta-lactamase. Examples of mammalian polypeptides include molecules such as, e.g., renin, a growth hormone, including human growth hormone, des-N-methionyl human growth hormone, and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; thyroxine; lipoproteins; alpha1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; leutinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor and Von Willebrands factor; anti-clotting factors such as Protein C; atrial naturietic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hematopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; a serum albumin such as human serum albumin; mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactanase; DNase; inhibin; activin; vascular endothelial growth factor; receptors for hormones or growth factors; integrin; thrombopoietin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-beta; platelet-derive growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-beta1, TGF-beta2, TGF-beta3, TGF-beta4, or TGF-beta5; insulin-like growth factor-I and -II (IGF-I and IGF-II); insulin-like growth factor binding proteins; CD proteins such as CD-3, CD-4, CD-8, and CD-19; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); somatotropins; interferons such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-15; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigens, such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; antibodies; and fragments of any of the above-listed polypeptides.

As used herein, the term "antibody" refers to full-length immunoglobins (IgA, IgD, IgE, IgG, IgM) and all isotypes thereof, humanized or chimeric antibodies, multispecific antibodies, CDR-modified antibodies, and antibody fragments thereof. Antibody fragments include Fab'$_2$, Fab, scFv single chain antibodies, and the like.

The preferred polypeptides of interest are those that are easily produced in bacterial cells with a minimum of proteolysis and a maximum in properly refolded or active material and need not be glycosylated for their intended utility. Examples of such mammalian polypeptides include antibodies (or fragments thereof), IGF-I, growth hormone, DNase, relaxin, growth hormone releasing factor, insulin, urokinase, immunotoxins, and antigens. Particularly preferred mammalian polypeptides include antibodies, IGF-I, and growth hormone.

A modified "host cell" is a cell in which a nucleic acid encoding the polypeptide of interest has been introduced. Alternatively the polypeptide of interest can be encoded by a gene that is part of the cell's genome, but for which regulatory sequences have been modified to provide for increased levels of expression.

Examples of host cells include, but are not limited to, bacterial organisms (bacteria), archaebacteria, simple single celled eukaryotes such as yeast and other fungi, plant cells, and animal cells. Suitable bacteria for this purpose include aerobic and facultative anaerobic bacteria, whether archaebacteria and eubacteria, especially eubacteria, and most preferably Enterobacteriaceae. Examples of useful bacteria include Escherichia, Enterobacter, Azotobacter, Erwinia, Bacillus, Pseudomonas, Klebsiella, Proteus, Salmonella, Serratia, Shigella, Rhizobia, Vitreoscilla, and Paracoccus. Suitable *E. coli* hosts include *E. coli* W3110 (ATCC 27,325), *E. coli* 294 (ATCC 31, 446), *E. coli* B, and *E. coli* X1776 (ATCC 31,537). These examples are illustrative rather than limiting. Mutant cells of any of the above-mentioned bacteria may also be employed. It is, of course, necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, *E. coli*, Serratia, or Salmonella species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon. *E. coli* strain W3110 is a particularly preferred parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins, with examples of such hosts including *E. coli* W3110 strain 1 A2, which has the complete genotype Δ fhuA; *E. coli* W3110 strain 9E4, which has the complete genotype Δ fhuA-ptr3; *E. coli* W3110 strain 27C7 (ATCC 55,244), which has the complete genotype Δ fhuA-ptr3 phoA-Δ-E15-Δ-(argF-lac) 169 ompT-Δ-degP41kan^r; *E. coli* W3110 strain 37D6, which has the complete genotype Δ fhuA-ptr3 phoA-Δ-E15-Δ-(argF-lac)169 ompT-Δ-degP41kan^r rbs7-Δ-ilvG; *E. coli* W3110 strain 40B 4, which is strain 37D6 with a non-kanamycin resistant degP deletion mutation; and an *E. coli* strain having mutant periplasmic protease disclosed in U.S. Pat. No. 4,946,783 issued Aug. 7, 1990. Examples of mammalian cells are COS-1 or CHO cells, HeLa cells, 293T (human kidney cells), mouse primary myoblasts, and NIH 3T3 cells. Examples of yeast species are *S. cerevisiae, Candida albicans, Candida utilis*, and *Phaffia rhodozyma*. Other suitable host cells are insect cells such as SF-9 cells (*Spodoptera frugiperda*).

Host cells grow under amenable culture conditions, i.e., appropriate conditions of temperature (generally around

25–37° C.), pH (generally pH 7–8), humidity (generally about 100%), oxygen, and nutrient availability including carbon/energy sources. As described herein, availability of oxygen and an energy source determine host cell growth rate.

As used herein, "large-scale" fermentation refers to fermentation in a fermentor that is at least approximately 1000 liters in volumetric capacity, i e., working volume, leaving adequate room for headspace. "Small-scale" fermentation refers generally to fermentation in a fermentor that is no more than approximately 100 liters in volumetric capacity, preferably no more than approximately 10 liters.

Recombinant Host Cells

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (herein "Sambrook et al., 1989"); *DNA Cloning: A Practical Approach*, Volumes I and II (D. N. Glover ed. 1985); *Oligonucleotide Synthesis* (M. J. Gait ed. 1984); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1985); *Transcription And Translation* (B. D. Hames & S. J. Higgins, eds. 1984); *Animal Cell Culture* (R. I. Freshney, ed. 1986); *Immobilized Cells And Enzymes* (MRL Press, 1986); *A Practical Guide To Molecular Cloning* (B. Perbel, 1984); *Current Protocols in Molecular Biology*, (F. M. Ausubel et al. eds. 1994). *Escherichia coli* and Salmonella (Neidhardt et al., ASM Press, 1996), particularly describes recombinant technology in bacteria.

A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

The polynucleotides herein may be flanked by natural regulatory (expression control) sequences, or may be associated with heterologous sequences, including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, introns, 5'- and 3'-non-coding regions, and the like. The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, and internucleotide modifications such as, for example, those with uncharged linkages (e.g ., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.). Polynucleotides may contain one or more additional covalently linked moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (e.g., acridine, psoralen, etc.), chelators (e.g., metals, radioactive metals, iron, oxidative metals, etc.), and alkylators. The polynucleotides may be derivatized by formation of a methyl or ethyl phosphotriester or an alkyl phosphoramidate linkage. Furthermore, the polynucleotides herein may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, i.e., the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence for a protein may include a start codon (usually ATG) and a stop codon.

The term "gene", also called a "structural gene" means a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed. Some genes, which are not structural genes, may be transcribed from DNA to RNA, but are not translated into a polypeptide sequence. Other genes may function as regulators of structural genes or as regulators of DNA transcription.

The terms "express" and "expression" mean allowing or causing the information in a gene or DNA sequence to become manifest, for example producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as a protein. The expression product itself, e.g., the resulting protein, may also be said to be "expressed" by the cell. An expression product can be characterized as intracellular, extracellular or secreted. The term "intracellular" means something that is inside a cell. The term "extracellular" means something that is outside a cell. A substance is "secreted" by a cell if it appears in significant measure outside the cell, from somewhere on or inside the cell.

The term "expression system" means a host cell and compatible vector under suitable conditions, e.g,. for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell. In a specific embodiment, the recombinant protein is expressed in *E coli* host cells.

The term "heterologous" refers to a combination of elements not naturally occurring. For example, heterologous DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell. A heterologous expression regulatory element is such an element operatively associated with a different gene than the one it is operatively associated with in nature. In the context of the present invention, a gene encoding a protein of interest in heterologous to the vector DNA in which it is inserted for cloning or expression, and it is heterologous to a host cell containing such a vector, in which it is expressed, e.g., an *E. coli* cell.

The term "transfection" means the introduction of a foreign nucleic acid into a cell. The term "transformation" means the introduction of a "foreign" (i.e., extrinsic or extracellular) gene, DNA or RNA sequence to a host cell, so that the host cell will replicate the DNA and express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme coded by the introduced gene or sequence. The introduced gene or sequence may also be called a "cloned" or "foreign" gene or sequence, and may include regulatory or control sequences, such as start, stop, promoter, signal, secretion, or other sequences used by a cell's genetic machinery. The gene or sequence may include nonfunctional sequences or sequences with no known function. DNA may be introduced either as an extrachromosomal element or by chromosomal integration, and a host cell tat receives and expresses introduced DNA or RNA has been "transformed" and is a "transformant" or a "clone." The

DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or cells of a different genus or species.

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook et al., supra, is generally used for bacterial cells that contain substantial cell-wall barriers. Another method for transformation employs polyethylene glycol/DMSO, as described in Chung and Miller (Nucleic Acids Res. 1988, 16:3580). Yet another method is the use of the technique termed electroporation.

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (e.g., a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (e.g., transcription and translation) of the introduced sequence. Vectors include plasmids, phages, viruses, etc.; they are discussed in greater detail below.

Vectors typically comprise the DNA of a transmissible agent, into which foreign DNA is inserted. A common way to insert one segment of DNA into another segment of DNA involves the use of enzymes called restriction enzymes that cleave DNA at specific sites (specific groups of nucleotides) called restriction sites. A "cassette" refers to a DNA coding sequence or segment of DNA that codes for an expression product that can be inserted into a vector at defined restriction sites. The cassette restriction sites are designed to ensure insertion of the cassette in the proper reading frame. Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA having inserted or added DNA, such as an expression vector, can also be called a "DNA construct." A common type of vector is a "plasmid", which generally is a self-contained molecule of double-stranded DNA, usually of bacterial origin, that can readily accept additional (foreign) DNA and that can readily be introduced into a suitable host cell. A plasmid vector often contains coding DNA and promoter DNA and has one or more restriction sites suitable for inserting foreign DNA. Coding DNA is a DNA sequence that encodes a particular amino acid sequence for a particular protein or enzyme. Promoter DNA is a DNA sequence that initiates, regulates, or otherwise mediates or controls the expression of the coding DNA. Promoter DNA and coding DNA may be from the same gene or from different genes, and may be from the same or different organisms.

A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Clonetech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, Wis.), pRSET or pREP plasmids (Invitrogen, San Diego, Calif.), or pMAL plasmids (New England Biolabs, Beverly, Mass.), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, e.g., antibiotic resistance, and one or more expression cassettes.

A wide variety of host/expression vector combinations (i.e., expression systems) may be employed in expressing the proteins of interest. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include known bacterial plasmids, e.g., *E. coli* plasmids col E1, pCR1, pBR322, pMal-C2, pET, pGEX (Smith et al., Gene 67:31–40, 1988), pMB9 and their derivatives, plasmids such as RP4; phage DNAS, e.g., the numerous derivatives of phage 1, e.g., NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2 m plasmid or derivatives thereof; vectors derived from combinations of plasmids and phage DNAs, such as plasmids that have been modified to employ phage DNA or other expression control sequences; and the like.

Yeast expression systems can also be used according to the invention to express any protein of interest. For example, the non-fusion pYES2 vector (XbaI, SphI, ShoI, NotI, GstXI, EcoRI, BstXI, BamH1, SacI, Kpn1, and HindIII cloning sit; Invitrogen) or the fusion pYESHisA, B, C (XbaI, SphI, ShoI, NotI, BstXI, EcoRI, BamH1, SacI, KpnI, and HindIII cloning site, N-terminal peptide purified with ProBond resin and cleaved with enterokinase; Invitrogen), to mention just two, can be employed according to the invention.

The host cells can inherently also harbor the polypeptide of interest. For example, alkaline phosphatase is a protein that is homologous to *E. coli* and can be induced without any further transfection of the cell with vector DNA. For heterologous polypeptides such as, e.g., antibody and growth hormone, the heterologous nucleic acid (e.g., cDNA) is suitably inserted into a replicable vector for expression in the culture medium under the control of a suitable promoter. As noted above, many vectors are available for this purpose, and selection of the appropriate vector will depend mainly on the size of the nucleic acid to be inserted into the vector and the particular host cell to be transformed with the vector. Each vector contains various components depending on its f unction (amplification of DNA or expression of DNA) and the particular host cell with which it is compatible. The vector components for bacterial transformation generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, and a promoter.

The DNA encoding the polypeptide of interest herein may be expressed not only directly, but also as a fusion with another polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the polypeptide DNA that is inserted into the vector. The heterologous signal sequence selected should be one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For bacterial host cells that do not recognize and process the native polypeptide signal sequence, the signal sequence is substituted by a bacterial signal sequence selected, for example, from the group consisting of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria.

Expression and cloning vectors also generally contain a selection gene, also termed a selectable marker. This gene

encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli. One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen.

The expression vector for producing a heterologous polypeptide also contains an inducible promoter that is recognized by the host organism and is operably linked to the nucleic acid encoding the polypeptide of interest. Promoters suitable for use with bacterial hosts include the beta-lactamase and lactose promoter systems (Chang et al., Nature 1978, 275:615; Goeddel et al., Nature 1979, 281:544), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res. 1980, 8:4057 and EPO 36,776) and hybrid promoters such as the tac promoter (deBoer et al., Proc. Natl. Acad. Sci. USA 1983, 80:21–25). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding the polypeptide of interest (Siebenlist et al., Cell 1980, 20:269) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the polypeptide of interest. The promoter can be removed from the bacterial source DNA by restriction enzyme digestion and inserted into the vector containing the desired DNA.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

An "inducible promoter" or "regulated promoter" is a promoter that regulates expression in response to a stimulus. The promoter may be bound by a transcription regulatory protein, e.g., a repressor or an activator, which represses or activates gene expression, respectively. The repressor or activator protein in turn is responsive to the stimulus, such as the presence or absence of a nutrient, such as lactose, a nutrient such as phosphate, a toxin, such as a heavy metal, acidic pH, increased temperature, or some other environmental signal.

A coding sequence is "under the control of" or "operatively associated with" transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced (if it contains introns) and translated into the protein encoded by the coding sequence.

Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) or other strains, and successful transformants are selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Sanger et al., Proc. Natl. Acad. Sci. USA 1977, 74:5463–5467 or Messing et al., Nucleic Acids Res. 1981, 9:309), or by the method of Maxam et al. (Methods in Enzymology 1980, 65:499).

Host cells are transformed with the above-described expression vectors of this invention and cultured in conventional nutrient media modified as appropriate for the promoter utilized.

Inducible Expression Systems

In general, plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used in connection with bacterial hosts. The vector ordinarily carries a replication site, as well as marking sequences that are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., Gene 1977, 2:95). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid or phage, also generally contains, or is modified to contain, promoters that can be used by the microbial organism for expression of the selectable marker genes.

Thus, expression of the polypeptide of interest may be controlled by any inducible promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters that may be used to control gene expression include, but are not limited to, prokaryotic expression promoters such as the beta-lactamase promoter (Villa-Komaroff, et al., Proc. Natl. Acad. Sci. USA 1978, 75:3727–3731), or the tac promoter (DeBoer, et al., Proc. Natl. Acad. Sci. USA 1983, 80:21–25; see also Sheibani, Prep. Biochem. Biotechnol. 1999, 29:77; "Useful proteins from recombinant bacteria" in Scientific American, 242:74–94, 1980; Gossen et al., Curr. Opin. Biotechnol. 1994, 5:516). In a specific embodiment, a phoA promoter provides for regulated expression. Expression systems have been described for industrial Gram-positive bacteria, such as Bacillus, Clostridium, Lactococcus, Lactobacillus, Staphylococcus and Streptococcus based primarily on the capacity of these bacteria to utilize specific sugars or to secrete autoinducing peptides that are involved in quorum sensing (de Vos et al., Curr. Opin. Biotechnol. 1997, 8:547), particularly for lactic acid bacteria (de Vos, Curr. Opin. Microbiol. 1999, 2:289; Djordjevic and Klaenhammer, Mol. Biotechnol. 1998, 9:127). Other expression systems include, but are by no means limited to, the tryptophan promoter (Chevalet et al., Biotechnol. Bioeng. 2000, 69:351), the *E. coli* Ntr-system (Schroeckh et al., J. Biotechnol. 1999, 75:241); the PalkBFGHJKL promoter (Staijen et al., J. Bacteriol. 1999, 181:1610); the *E. coli* araBAD promoter/araC regulator system (Newman and Fuqua, Gene 1999, 227:197); tetracycline-regulated systems (Liu et al., Biotechniques 1998, 24: 624; Gallia and Khalili, Oncogene 1998, 16:1879); the VanS-VanR two-component

regulatory system (Haldimann et al., J. Bacteriol. 1997, 179:5903); a potassium-regulated system (Treuner-Lange et al., J. Bacteriol. 1997, 179:4501); a pH-inducible system (San et al., Ann. NY Acad. Sci. 1994, 721:268), a peroxide/ascorbate-inducible system (Bishai et al., J. Bacteriol. 1994, 176:2914); systems based on the T7 promoter (see Lama and Carrasco, Biochem. Biophys. Res. Commun. 1992, 188:972); antibiotic-inducible systems (see Nielsen et al., Mol. Microbiol. 1991, 5:1961); and other regulated systems (see Alksne and Rasmussen, J. Bacteriol. 1997, 179:2006; Everett et al., Microbiology 1995, 141:419).

Inducible promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter are also well known.

Fermentations

Various larger-scale fed-batch fermentations are available for production of recombinant proteins. Larger fermentations have at least 1000 liters of capacity, preferably about 1000 to 100,000 liters of capacity, i.e., working volume, leaving adequate room for headspace. These fermentors use agitator impellers or other suitable means to distribute oxygen and nutrients, especially glucose (the preferred carbon/energy source). Small-scale fermentation refers generally to fermentation in a fermentor that is no more than approximately 100 liters in volumetric capacity, preferably no more than approximately 10 liters.

Host cells used to produce the polypeptide of interest of this invention are cultured in suitable media in which the promoter can be constitutively or artificially induced as described generally, e.g, in Sambrook et al., supra. Examples of suitable media are given below in the example section.

Any necessary supplements besides carbon, nitrogen, and inorganic phosphate sources may also be included at appropriate concentrations introduced alone or as a mixture with another supplement or medium such as a complex nitrogen source.

Initially, prior to expression of the polypeptide of interest, the host cells inoculated into the fermentor are grown under favorable growth conditions, e.g., with all of the available oxygen and carbon/energy sources (or, preferably, source), along with essential nutrients and pH control, necessary for logarithmic growth. In accordance with the invention, these conditions are maintained, e.g., by feeding concentrated glucose at a rate that controls dissolved oxygen content at a set point, until the host cells expand in culture to the desired number or cell density.

After reaching target cell density, two manipulations of the fermentation occur. The first is to provide the signal to induce expression of the polypeptide of interest, e.g., by depleting phosphate levels as exemplified infra.

The second manipulation (which can result from the first) is to downshift or reduce the host cell metabolic rate. Since during logarithmic growth the metabolic rate is directly proportional to availability of oxygen and a carbon/energy source, reducing the levels of available oxygen or carbon/energy sources, or both, will reduce metabolic rate. Manipulation of fermentor operating parameters, such as agitation rate or back pressure, as well as reducing O_2 pressure, modulates available oxygen levels. Reducing concentration or delivery rate, or both, of the carbon/energy source(s) has similar effect. Furthermore, depending on the nature of the expression system, induction of expression can lead to a dramatic decrease in metabolic rate. Finally, upon reaching maximum cell density, growth stops or the rate decreases dramatically.

As discussed above, reduction in host cell metabolic rate results in more controlled expression of the polypeptide of interest, which permits folding and protein assembly to occur.

Gene expression may be measured in a sample directly, for example, by conventional Northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA 1980, 77: 5201–5205). Various labels may be employed, most commonly radioisotopes, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like.

The polypeptide of interest preferably is recovered from the periplasm or culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly expressed without a secretory signal. Alternatively, the cells or portions thereof may be used as biocatalysts or for other functions without substantial purification.

It is often preferred to purify the polypeptide of interest from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the polypeptide of interest. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions may then be separated if necessary. The polypeptide may then be purified from the soluble protein fraction and from the membrane fraction of the culture lysate, depending on whether the polypeptide is membrane bound, is soluble, or is present in an aggregated form. The polypeptide thereafter is solubilized and folded, if necessary, and is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; and gel filtration using, for example, Sephadex G-75.

EXAMPLES

The following examples illustrate without limiting the invention.

Example 1

Anti-CD18 Fermentation

Materials and Methods

Fermentation

The host used in these fermentations was a derivative of *E. coli* W3110, designated 49A5. The complete genotype of 49A5 is W3110 ΔfhuA phoAΔAE15Δ(argF-lac)169deoC degP41 (ΔPstI-Kan^r) IN (rrnD-rrnE)1 ilvG2096(Val^r) ΔfucPΔmalE. The plasmid, pS1130, contains the genes from the light-chain fragment of anti-CD18 and a fragment of the heavy chain with a C-terminal leucine-zipper, both behind the phoA promoter and both preceded by a STII signal sequence. Consequently, upon induction of expression, the light-chain and heavy-chain fragments were synthesized and secreted into the periplasm where some fraction of the individual peptides fold and assemble to form an Fab'$_2$ with leucine zippers on the heavy chains.

For each 10-liter fermentation, a single vial containing 1.5 ml of culture in 10–15% DMSO was thawed into a 1L shake

flask containing 500 ml of LB medium supplemented with 0.5 ml of tetracycline solution (5 mg/ml) and 2.5 ml 1M sodium phosphate solution. This seed culture was grown for approximately 16 hours at 30° C. and was then used to inoculate a 10-liter fermentor.

The fermentor initially contained approximately 4.7 liters of medium containing approximately 3.5 g of glucose, 75 ml of 1M magnesium sulfate, 8 ml of a trace element solution (27 g/L ferric chloride hexahydrate, 8 g/L zinc sulphate heptahydrate, 7 g/L cobalt chloride hexahydrate, 7 g/L sodium molybdate, 8 g /L cupric sulfate pentahydrate, 2 g /L boric acid, 5 g/L manganese sulfate monohydrate, 10% hydrochloric acid), 8 ml of a 2.7% ferric chloride solution, 15 ml of a tetracycline solution (5 mg/ml in ethanol), 7.5 ml of Fermax Adjuvant 27 (or some equivalent anti-foaming agent), 1 bag of HCD salts (19.5 g potassium phosphate dibasic, 9.75 g sodium phosphate monobasic dihydrate, 37.5 g ammonium sulfate, 7.5 g sodium citrate dihydrate, 11.3 g potassium phosphate monobasic) and 150 g of NZ Amine A (a protein hydrolysate). Fermentations were performed at 30° C. with 10 slpm of air flow and were controlled at a pH of 7.0±0.2 (although occasional excursions beyond this range occurred in some cases). The fermentor back pressure and agitation rate were varied to manipulate the oxygen transfer rate in the fermentor and, consequently, control the cellular respiration rate.

Following inoculation of the fermentor with the cell-containing medium from the shake flask, the culture was grown in the fermentor to high cell densities using a computer-based algorithm to feed a concentrated glucose solution to the fermentor. Ammonium hydroxide (58% solution) and sulfuric acid (24% solution) were also fed to the fermentor as needed to control pH. Further additions of anti-foam were also used in some cases to control foaming. When the culture reached a cell density of approximately 40 OD_{550}, an additional 75 ml of 1M magnesium sulfate was added to the fermentor. Additionally, a concentrated salt feed (10 g/L ammonium sulfate, 26 g/L potassium phosphate dibasic, 13 g/L sodium phosphate monobasic dihydrate, 2 g/L sodium citrate dihydrate, 15 g/L potassium phoshate monobasic, 8 ml/L 2.7% ferric chloride, 8 ml/L trace elements solution) was started at a rate of 1.9 ml/min when the culture reached approximately 20 OD_{550} and continued until approximately 940 ml were added to the fermentation. Fermentations were typically continued for 72–80 hours.

During the fermentation, once the dissolved oxygen setpoint for the fermentation was reached, the concentrated glucose solution was fed based on the dissolved oxygen probe signal in order to control the dissolved oxygen concentration at the setpoint without excess glucose feeding. Consequently, in this control scheme, manipulations of fermentor operating parameters such as the agitation rate or back pressure, which affect the oxygen transfer capacity in the fermentation, correspondingly manipulate the oxygen uptake rate or metabolic rate of the cells.

A mass spectrometer was used to monitor the composition of the off-gas from the fermentations and enable the calculation of the oxygen uptake and carbon dioxide evolution rates in the fermentations.

Product Assays

To assess the quantity of antibody product in the fermentations, a number of assays were used. To measure assembled anti-CD18 Fab'_2-leucine zipper, a cation exchange HPLC (high performance liquid chromatography) assay was used. To prepare cell samples for this assay, fermentation broth was first diluted in phosphate-buffered saline to a concentration of 20 OD_{550}. One ml of this diluted broth was then centrifuged for 15 minutes at approximately 15,000×g and the remaining supernatant was discarded, leaving a cell pellet for the HPLC analysis. This pellet was then frozen at −20° C. to −70° C. until needed for the assay. The frozen pellets were resuspended in a lysis buffer containing 500 μl of 100 mM (or 200 mM) TRIS buffer at pH 8, 20 μl of 6 mg/ml lysozyme in water (freshly prepared), and 10 μl of 100 mM EDTA. The samples were sonicated for 10 pulses, and then incubated typically on ice for at least thirty minutes prior to further analysis. In some cases, a second round of sonication may then be performed. The samples were then centrifuged again for fifteen minutes at approximately 15,000×g to obtain the soluble fraction of the lysate (in the supernatant). Samples were diluted at least 1:1 and 250 μl were loaded onto a CsX column on a Hewlett-Packard 1090 HPLC system. Samples were eluted using a gradient of 5 to 50 mM sodium phosphate (pH 7.0) over fourteen minutes, and peaks were monitored using UV absorbance at 278 nm. The peak containing anti-CD18 Fab'2-leucine zipper was identified and quantified by comparison with purified standards.

Results

A series of anti-CD18 Fab'_2 fermentations were run in which varying oxygen uptake rate profiles were used (FIG. 1). The control used a constant with a previously favorable oxygen uptake rate. The x-axis shows the approximate oxygen uptake rates during the growth phase and at the end of the fermentation. The case with the single number of 3.5 represents an unshifted control run at approximately 3.5 mmol/1/min O_2. The controls represent an average of nine fermentations run using similar conditions to the shifted cases with the exception that no attempts were made to shift the OUR resulting in roughly constant OUR's of 3.5.

The actual oxygen uptake rate profiles from the fermentations shown in FIG. 1 are displayed and are grouped according to titer (FIG. 2). The runs in which the titer exceeded 1000 mg/L are shown in four runs (#1–4) recorded with a thin solid line, the runs in which the titer was between 800–900 mg/L are shown in four runs (#5–8) recorded with a dotted line, and the unshifted control run is shown in the run (#9) recorded with a heavy solid line. These results strongly support the hypothesis that increased down-shifts of the oxygen uptake rates significantly increase the fermentation yield of anti-CD18 Fab'2.

To further investigate the cause of this effect, the titer assay results for these various runs are shown as a function of fermentation time (FIG. 3). These results suggest that in the cases with the largest OUR shifts, anti-CD18 yield is increased as a result of an extended period of product formation.

Example 2

Anti-VEGF Fermentation

Materials and Methods

Fermentation

The organism used for these fermentations was 43H1 W3110 ΔfhuA phoAΔE15 Δ(argF-lac)169ptrA ΔompT degP41 (ΔPstI-Kan^r) IN (rrnD-rrnE)1 ilvG209(Val^r) prc::kan prc suppressor. The plasmid used in these runs was Y0317tet20 and confers resistance to ampicillin and tetracycline. Anti-VEGF Fab was expressed from the phoA promoter. For the standard protocol, the fermenter conditions did not change with time. For the oxygen use rate (OUR) shifted runs, the agitation and back pressure were

gradually decreased from 1000 RPM and 1.0 bar to 600 RPM and 0.3 bar respectively.

The standard and "OUR" fermentation protocols are summarized in Table 1.

TABLE 1

OUR Shift and Standard Protocols

Parameter	Standard	OUR Shifted
Temperature	30° C.	30° C.
Airflow	20 L/min	20 L/min
Back pressure	1.0 bar constant	1.0 start decreasing to 0.3 bar
Agitation Rate	constant	1000 RPM decreasing to 600
Salt Feed	650 RPM constant high cell density salts*	high cell density salts* with yeast extract (100 g)

*High cell density salts is a feed consisting of inorganic salts yielding between 200–300 OD_{550} units. Extra yeast extract without the OUR shift protocol decreases the yield of the product.

Product Sample Preparation and Product Assay

One-milliliter samples of fermentation broth were taken every two hours and frozen on dry ice. Samples were stored long term at −20° C. The samples were subsequently thawed and diluted 6× into extraction buffer (10 mM TrisCl, pH6.8, 1 mM EDTA, 0.2 mg/ml lysozyme, and 5 mM iodoacetamide) and vortexed. After ten minutes on ice, 1/100 volume of 1 M $MgSO_4$ and 1/100 volume of 2 mg/ml DNAse were added and the samples vortexed again. After another ten-minute incubation on ice, the samples were sonicated for ten seconds (Branson sonifier, microtip probe, setting 4, pulsed) and placed back on ice. Sonication was repeated for a total of two rounds. The samples were then centrifuged for twenty minutes at 4° C. at 16,000×g. The resulting supernatants were then analyzed by affinity chromatography using VEGF immobilized on an HPLC resin (Poros AL). The VEGF column was loaded and equilibrated in phosphate-buffered saline and the product eluted in 12 mM HCl with 150 mM NaCl. The product was quantitated by measuring the A_{280} of the samples and comparing to a standard curve generated by spiking purified anti-VEGF into fermentation broth containing either anti-CD18 or Apo2L.

Results

Observations for anti-VEGF Fab fermentations were similar to those with anti-CD18 Fab'$_2$. Titer profiles for a series of anti-VEGF fermentations with and without OUR shifts are shown in FIG. **4**. These data demonstrate a statistically significant improvement in titer as a result of the OUR shifts compared to the standard protocol (See Table 2).

TABLE 2

Titer profiles for OUR Shifts vs Standard Protocols

	OUR Shift (mg/L)	Standard (mg/L)
RUN 1	1107.6	805.4
RUN 2	999.5	552.5
RUN 3	956.4	736.9
Average	1021.2	698.3
Standard Deviation	77.9	130.8
t-test	0.01	

The corresponding OUR profiles for these runs are shown in FIG. **5**.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from

the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all values are approximate, and are provided for description.

Patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

What is claimed is:

1. A method for increasing product yield of a properly folded polypeptide of interest produced by recombinant host cells, wherein expression of the polypelptide by the recombinant host cells is regulated by an inducible system, which method comprises

(a) culturing the recombinant host cells under conditions of high metabolic and growth rate; and

(b) reducing the metabolic rate of the cultured recombinant host cells at the time of induction of polypeptide expression, wherein reducing the metabolic rate comprises reducing the feed rate of a carbon/energy source, or reducing the amount of available oxygen, or both, and wherein the reduction in metabolic rate results in increased yield of properly folded polypeptide.

2. The method according to claim **1**, wherein reducing the metabolic rate comprises decreasing available oxygen to the host cells.

3. The method according to claim **1**, wherein reducing the metabolic rate comprises decreasing available carbon/energy sources to the host cells.

4. The method according to claim **3**, wherein the carbon/energy source is glucose.

5. The method according to claim **1**, wherein reducing the metabolic rate comprises decreasing both the oxygen transfer rate and available carbon/energy source to the host cells.

6. The method according to claim **1**, wherein the metabolic rate is reduced by about half in step (b).

7. The method according to claim **1**, which comprises growing the cells to maximum density in step (a).

8. The method according to claim **7**, wherein the metabolic rate is reduced by about half in step (b).

9. The method according to claim **1**, wherein the recombinant host cell is a bacterial cell selected from the group consisting of *E. coli* and Salmonella.

10. The method according to claim **1**, wherein the inducible system is a phosphate depletion inducible system.

11. The method according to claim **1**, wherein the polypeptide is assembled in the host cell.

12. The method according to claim **9**, wherein the polypeptide is secreted into the periplasm of the host cell.

13. The method according to claim **1**, wherein the polypeptide is an antibody.

14. The method according to claim **1**, wherein the polypeptide is selected from the group consisting of an Fab'$_2$ antibody and an Fab antibody or other form of antibody.

15. The method according to claim **1**, wherein the metabolic and growth rate of the host cells is maximized in step (a).

16. A method of increasing product yield of a properly folded antibody, growth factor, or mammalian protease produced by a recombinant *E. coli* host cell, wherein expression of the antibody, growth factor, or protease is regulated by an inducible system, which method comprises

(a) culturing host cells under conditions of high metabolic and growth rate; and

(b) reducing metabolic rate of the recombinant host cells at the time of induction of antibody, growth factor, or protease expression.

17. The method according to claim **16**, wherein the antibody is an Fab'$_2$ antibody.

18. The method according to claim **16**, wherein the antibody is an Fab antibody.

19. The method according to claim **16**, wherein reducing the metabolic rate comprises decreasing available oxygen to the host cells.

20. The method according to claim **16**, wherein reducing the metabolic rate comprises decreasing available carbon/energy sources to the host cells.

21. The method according to claim **19**, wherein the carbon/energy source is glucose.

22. The method according to claim **16**, wherein the metabolic rate is reduced by about half in step (b).

23. The method according to claim **16**, wherein the inducible system is a phosphate depletion inducible system.

24. The method according to claim **16**, wherein the metabolic and growth rate of the host cells is maximized in step (a).

25. A method for increasing product yield of a properly folded mammalian polypeptide of interest produced by recombinant host cells, wherein expression of the polypeptide by the recombinant host cells is regulated by an inducible system, which method comprises

(a) culturing the recombinant host cells under conditions of high metabolic and growth rate; and

(b) reducing metabolic rate of the recombinant host cells at the time of induction of polypeptide expression.

26. The method according to claim **25**, wherein reducing the metabolic rate comprises decreasing available oxygen to the host cells.

27. The method according to claim **25**, wherein reducing the metabolic rate comprises decreasing available carbon/energy sources to the host cells.

28. The method according to claim **25**, wherein the carbon/energy source is glucose.

29. The method according to claim **25**, wherein reducing the metabolic rate comprises decreasing both the oxygen transfer rate and available carbon/energy source to the host cells.

30. The method according to claim **25**, wherein the metabolic rate is reduced by about half in step (b).

31. The method according to claim **25**, which comprises growing the cells to maximum density in step (a).

32. The method according to claim **31**, wherein the metabolic rate is reduced by about half in step (b).

33. The method according to claim **25**, wherein the recombinant host cell is a bacterial cell selected from the group consisting of *E. coli* and Salmonella.

34. The method according to claim **25**, wherein the inducible system is a phosphate depletion inducible system.

35. The method according to claim **25**, wherein the polypeptide is assembled in the host cell.

36. The method according to claim **33**, wherein the polypeptide is secreted into the periplasm of the host cell.

37. The method according to claim **25** wherein the polypeptide is an antibody.

38. The method according to claim **25**, wherein the polypeptide is selected from the group consisting of an Fab'$_2$ antibody and an Fab antibody or other form of antibody.

39. The method according to claim **25**, wherein the metabolic and growth rate of the host cells is maximized in step (a).

* * * * *

EXHIBIT Z

US007390660B2

(12) **United States Patent**
Behrendt et al.

(10) **Patent No.: US 7,390,660 B2**
(45) **Date of Patent: Jun. 24, 2008**

(54) **METHODS FOR GROWING MAMMALIAN CELLS IN VITRO**

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(58) **Field of Classification Search** 435/325, 435/326

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,328,844 A 7/1994 Moore
5,719,050 A * 2/1998 Hashimoto et al. 435/69.1
6,458,565 B1 * 10/2002 Cunningham et al. 435/70.3
6,900,056 B2 * 5/2005 Lee et al. 435/404
2002/0042132 A1 * 4/2002 Gardner et al. 435/388
2003/0096402 A1 * 5/2003 Lee et al. 435/325

FOREIGN PATENT DOCUMENTS

JP 405095783 A * 4/1993
WO WO 93/00423 A1 1/1993
WO WO98/41611 9/1998
WO WO 01/94552 A2 12/2001

OTHER PUBLICATIONS

Chen et al., Biotechnol. Bioeng., 72, pp. 55-61 (2001).
Colosimo et al., BioTechniques, 29, pp. 314-331 (2000).
Comer et al., Cytotechnology, 3, pp. 295-299 (1990).
Glacken et al., Biotechnol. Bioeng., 28, pp. 1376-1389 (1986).
Hu et al., Dev. Biol. Stand., 66, pp. 279-290 (1987).
Irani et al., Biotechnol. Bioeng., 66, pp. 238-246 (1999).
Xie et al., Cytotechnology, 15, pp. 17-29 (1994).
Hassell et al., J. Cell Sci., 96, pp. 501-508 (1990).
Europa et al., Biotechnol. Bioeng., 67, pp. 25-34 (2000).
Altamirano et al., Biotechnol. Prog., 16, pp. 69-75 (2000).
Xie et al., Trends in Biotechnology, 15, pp. 109-113 (1997).
Akshay Goel, et al., Biotechnol. Prog 4, pp. 380-385 (1995).
Shimomura, Y. et al, *Abstract of the 53rd Congress of Japan Society of Nut. & Food Sci*, (1999) 57.
Faiz-ur-Rahman, A.T.M. et al, *Planta*, 118 (1974) 211-224.
Toftegaard Nielsen, T. et al, *Scan. J. Lab. Invest.*, 40 (1980) 575-580.
Zammit, V.A., *FEBS Letters*, 108 (1979) 193-196.
Hencsey, Z., et al., Acta Microbiol. Hung., 43, pp. 359-370 (1996).
Dhir, S., et al., Biotechnol. Bioeng., 67, pp. 197-205 (2000).
Miller, W.M., et al., Biotechnol. Bioeng., 67, pp. 853-871 (2000).
Pörtner, R., and Schäfer, T., J. Biotechnol., 49, pp. 119-135 (1996).
Simonson, L., et al., Cytobios, 77, pp. 159-165 (1994).
Kromenaker, S., and Srienc., F., J. Biotechnol., 34, pp. 13-34 (1994).

* cited by examiner

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(57) **ABSTRACT**

The invention relates to a method for reducing glucose consumption during cultivation of animal cells, which comprises cultivating animal cells in the presence of a bi- or tricarbonic acid or a salt thereof at a concentration of about 1 to 50 mmol/l.

6 Claims, No Drawings

METHODS FOR GROWING MAMMALIAN CELLS IN VITRO

FIELD OF THE INVENTION

The invention provides a method for reducing lactate formation and/or glucose consumption in mammalian cell cultures by the addition of a bi- or tricarbonic acid, e.g. citric acid.

BACKGROUND OF THE INVENTION

Optimization strategies of cell culture processes aim at maximizing the longevity of cell culture (Bibila, T. A., and Robinson, D. K., Biotechnol. Prog. 11 (1995) 1-13). The final integrated number of viable cells over cultivation time is often used as a measure of cultivation success and is positively correlated with product formation. As used herein, this integral is defined as CTI (CTI=Cell density Time Integral).

Lactate is a major waste product formed during the cultivation of mammalian cells. Under typical culture conditions, the cells consume glucose in great excess and metabolize it mainly to lactate. The accumulation of lactate affects cell growth, CTI and protein production adversely as a result of pH and/or pH adjustment by alkali (Chang, Y. H. D., et al., Biotechnol. Bioeng. 47 (1995) 319-326); Omasa, T., et al., Biotechnol. Bioeng. 39 (1992) 556-565 and Chen, K., et al., Biotechnol. Bioeng. 72 (2001) 55-62).

There have been a lot of attempts to reduce lactate formation. It was suggested by Glacken, M. W., et al., (Biotechnol. Bioeng. 28 (1986) 1376-1389); Hu, W. S., et al., (Dev. Biol. Stand. 66(1987) 279-290); and Xie, L., and Wang, D. I. C., Cytotechnology 15 (1994) 17-29) to grow mammalian cells at low glucose concentrations with dynamic controlled feeding with glucose. The idea was to achieve a metabolic shift from high glucose/lactate flux to a low glucose/lactate flux. However, such methods require adaptations of the cells and need carefully designed control mechanisms of feeding. They are therefore complicated and difficult to perform (U.S. Pat. No. 6,156,570).

Other methods for reducing lactate formation are based on genetic engineering means. One method is described by Chen, K., et al., Biotechnol. Bioeng. 72 (2001) 55-62) suggesting manipulation of the metabolic pathway for lactate in the mammalian cells by inactivation of at least one copy of lactate dehydrogenase genes in the cells. Another method is described by Irani, N., et al., (J. Biotechnol. 66 (1999) 238-246), which introduces a pyruvate carboxylase gene into the host cell genome. It is assumed that the conversion of pyruvate to lactate is reduced and therefore the longevity of the cell culture is improved.

The addition of ferric citrate as a substituent for transferring in serum-free media for the cultivation of mammalian cells has been known for a long time (cf., e.g., Toyoda, K., and Inouye, K., Agric. Biol. Chem. 55 (1991) 1631-1633; Franek, F., and Dolnikova, J., Cytotechnology 7 (1991) 33-38; Kovar, J., and Franek, F., Exp. Cell Res. 182 (1989) 358-369; Schneider, Y. J., J. Immunol. Meth. 116 (1989) 65-77; and Kovar, J., Hybridoma 7 (1988) 255-263).

SUMMARY OF THE INVENTION

It has surprisingly been found that the addition of one or more bi- or tricarbonic acids inhibits the consumption of glucose and/or the formation of lactate from glucose considerably and therefore, improves cell density and cell viability during mammalian cell cultivation. Based on these findings,

the yield of a protein of interest (POI) which is produced by such a cell cultivation increases considerably using the method according to the present invention. Further, the addition of bi- or tricarbonic acid reduces the amount of alkali which is needed to maintain a constant pH value from about 50% to about 70%. Accordingly, the present invention relates to a method for reduction of glucose consumption and/or lactate production during cultivation of animal cells in vitro. The cultivation is performed in the presence of one or more bi- or tricarbonic acids or their salts, such as oxoglutaric acid, succinic acid, fumaric acid, malic acid, ketoglutaric acid or citric acid or combinations thereof at a concentration of about 1 to 50 mmol/l. Specifically, where the di- or tricarbonic acid or salt is citric acid or citrate, this amount of citric acid or citrate is not bound in chelate complex with iron or another transition metal ion.

In a preferred embodiment of the invention, the animal cells are mammalian cells, preferably hybridoma or myeloma cells, CHO, NS0, BHK, or HeLa cells which produce monoclonal antibodies or proteinaceous hormones.

In a further preferred embodiment of the invention, the cells are cultivated in a fed batch, batch perfusion, dialysis, solid state, or continuous fermentation, preferably over a time period of 10 to 20 days.

DETAILED DESCRIPTION OF THE INVENTION

According to the invention, the specific rate of glucose consumption (μg/10^6 cells×day) is reduced to at least about 40%, preferably from about 40 to about 60%, in relation to a fermentation process using a method in which no uncomplexed citrate is present. The specific rate of lactate production (μg/10^6 cells×day) is reduced to at least about 50%, preferably from about 50 to about 70%, in relation to a fermentation process using a method in which no uncomplexed citrate is present.

Accumulation of lactate in the cell culture medium can inhibit cell growth and the POI production during cell cultivation. The growth-inhibitory lactate concentration varies with the cell line and the process. The lactate concentration after a certain time in a cultivation process is a result of the specific production rate of lactate and the CTI. This invention reduces the specific production rate of lactate, so that the period prolongs before the inhibitory concentration takes place, or in the best case, the concentration of lactate stays below the inhibitory concentration. However, this invention results in considerable increase of the CTI during the cultivation.

Cultivation of the cells is performed in a production dimension, i.e., in volumes of 10-10,000 l bioreactors. Such methods are described, for example, in Bibila, T. A., and Robinson, D. K., Biotechnol. Prog. 11 (1995) 1-13.

Preferably, the fermentation medium is a serum-free medium. Such media are widely described in the state of the art (see e.g. Murakami, H., Monoclonal Antibodies: Production and Application (1989) 107-141).

Bi- and tricarbonic acids are preferably added as an alkali metal or alkaline metal salt or as free acid at a concentration of about 1 to 50 mmol/l. This acid is preferably not bound to a chelate complex with iron or another transition metal. However, the medium may preferably contain an additional amount of a bi- and tricarbonic acid or a citrate salt thereof in a chelate complex with iron, which is added as an iron source of the serum-free medium. As previously stated, iron citrate is widely known as an additive to serum-free media as iron source should be, for example, transferrin.

The term "complexed bi- and tricarbonic acid" means an aqueous solution of stoichiometric amounts of the acid and iron ions which leads to complex formation within the law of mass action.

POI refers to any protein for which expression is desired. Preferably the term encompasses any recombinant form of a desired protein. Such proteins of interest are, for example, protein hormones like erythropoietin, or antibodies and the like. Such recombinant proteins are reviewed by, e.g., Hudson, P. J., and Souriau, C., Expert. Opin. Biol. Ther. 1 (2001) 845-855).

The mammalian cells are preferably recombinant cell lines like CHO cells or hybridoma or which myeloma cells are transformed with expression vectors capable of expressing such a POI. Such methods are well known in the art and reviewed by, e.g., Colosimo, A., et. al., Biotechniques 29 (2000) 314-331).

Fermentation in fed batch mode is preferably performed in stirred bioreactors for 4 to 10 days. The cell density is preferably between about 0.2 to about 10×10^6 cells/ml. PO_2 is preferably between about 15 to about 30% and pH between about 6.9 to about 7.3.

Fermentation in dialysis mode (Comer, M. J., et. al., Cytotechnology 3 (1990) 295-299) can be performed in stirred dialysis bioreactors for about 12 to about 16 days. The cell density is preferably between about 0.2 to about 30×10^6 cells/ml. PO_2 between about 15 to about 30% and pH between about 6.9 to about 7.3.

A common serum-free fermentation medium is used and a solution of concentrated nutrients is used for feeding.

The following examples and references are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

EXAMPLE 1

The cells of a myeloma cell line (Sp2/0) were thawed and expanded up to 2 L in spinner flasks over a period of approximately 14 days for the inoculation of a 10 L bioreactor. After 2 to 4 days the cells were split or transferred to a 100 L bioreactor and further cultivated for 2 to 4 days. The 100 L bioreactor served as inoculum for the 1000 L production bioreactor. For each inoculum step a starting cell density of about 0.2 to about 0.4×10^6 viable cells per mL was used.

The production bioreactor ran in a dialysis mode. The bioreactor was a stirred tank reactor with a working volume of about 900 to about 1300 L. Aeration was performed by sparging. The following process parameters were controlled: pH, temperature, pO^2, pressure and agitation rate. The bioreactor was equipped for dialysis mode with hollow fibre cartridges. The cartridges were connected to an external loop with a dialysis medium reservoir. The dialysis of the culture was started 2 to 4 days after inoculation. During fermentation the reservoir was repeatedly filled up with fresh dialysis medium. Some medium components were fed as separate sterile solutions to the bioreactor. These comprise glucose, amino acids, vitamins, and trace elements.

Fermentation was terminated after a maximum of 16 days.

The citrate was added to the fermentation medium and to the dialysis medium.

Results of Example 1

Tables 1 and 2 show the specific consumption rate of glucose and the CTI of runs with and without the addition of citrate (similar results can be found if fumarate is used). The fermentations were performed in the dialysis mode at the 1000 L scale. The addition of citrate to the media reduced the specific consumption rate for glucose about 44% and increased the CTI to about 205%. At the same time the specific production rate for lactate was reduced to about 40% (Tables 3 and 4).

During fermentation the specific production rate of the POI were nearly constant, therefore the amounts of POI comparatively increased with the CTI.

TABLE 1

Fermentations In Dialysis Mode Without Addition Of Citrate

Run No.	Specific consumption rate of glucose µg/10^6 cells x day	CTI Relative units
1	663	134
2	873	98
3	776	96
4	736	100
5	1424	72
Mean:	894	100

TABLE 2

Fermentations In Dialysis Mode With Addition Of 2.4 Mmol/L Citrate

Run No.	Specific consumption rate of glucose µg/10^6 cells x day	CTI Relative units
6	705	142
7	351	262
8	429	219
9	549	195
10	481	208
11	355	275
12	535	213
13	537	128
14	519	215
15	578	192
Mean:	504	205

By addition of citrate to the fermentation medium, the amount of alkali which is required to adjust a constant pH value during the cultivation was reduced to about 34% of the control (Tables 3 and 4).

TABLE 3

Fermentations In Dialysis Mode Without Addition Of Citrate

Run No.	Specific production rate of lactate µg/10^6 cells x day	Alkali Addition Relative units
1	506	110
2	746	111
3	574	59
4	595	77
5	1064	144
Mean:	697	100

TABLE 4

Fermentations In Dialysis Mode With Addition Of 2.4 Mmol/L Citrate

Run No.	Specific production rate of lactate µg/10^6 cells x day	Alkali addition Relative units
6	491	107
7	176	18

TABLE 4-continued

Fermentations In Dialysis Mode With Addition Of 2.4 Mmol/L Citrate

Run No.	Specific production rate of lactate µg/10^6 cells x day	Alkali addition Relative units
8	200	19
9	320	13
10	241	40
11	200	17
12	284	15
13	338	8
14	259	55
15	307	46
Mean:	282	34

EXAMPLE 2

The cells of a myeloma cell line were thawed (Sp2/0) and expanded up to 2 L in spinner flasks for the inoculation of a 10 L bioreactor.

The production bioreactor ran in a fed batch mode. The bioreactor was a stirred tank reactor with a working volume of from about 9 to about 13 L. Aeration was performed by sparging. The following process parameters were controlled: pH, temperature, pO2, pressure and agitation rate. Feeding of the culture was started 2 to 4 days after inoculation. Components including glucose, amino acids, vitamins, and trace elements were fed as separate sterile solutions to the bioreactor.

Fermentation was terminated after maximum of 10 days.

Citrate was added to the fermentation medium and the feeding medium.

Results of Example 2

Tables 5 and 6 show the specific production rate and the CTI of runs with and without the addition of citrate. The fermentations were performed in the feed batch mode at the 10 L scale. The addition of citrate to the media reduced the specific consumption rate for glucose to about 56% and increased the CTI to about 341%. At the same time the specific formation rate for lactate reduced to about 48%. This demonstrates the inhibition of the metabolic flux from glucose through glycolysis by the addition of citrate.

TABLE 5

Fermentations In Fed Batch Mode Without Addition Of Citrate

Run No.	Specific consumption rate of glucose µg/10^6 cells x day	Specific production rate of lactate µg/10^6 cells x day	CTI Relative units
1	1206	670	84
2	939	509	84
3	779	527	104
4	703	471	140
5	1022	714	75
6	948	727	62
7	839	665	90
8	1233	867	86
9	912	612	90
10	967	668	184
Mean:	955	643	100

TABLE 6

Fermentations In Fed Batch Mode With Addition Of 2.4 Mmol/L Citrate

Run No.	Specific consumption rate of glucose µg/10^6 cells x day	Specific production rate of lactate µg/10^6 cells x day	CTI Relative units
11	443	230	478
12	715	392	321
13	451	304	224
Mean:	536	309	341

What is claimed is:

1. A method for reducing glucose consumption during cultivation of CHO, myeloma, or hybridoma cells, comprising cultivating CHO, myeloma, or hybridoma cells in culture medium in the presence of citric acid or citrate wherein said citric acid or citrate is maintained at a concentration of about 1 to 50 mmol/l during cultivation and wherein said citric acid or citrate is not bound in a chelate complex with iron or another transition metal ion.

2. The method of claim 1, wherein the cells are myeloma cells.

3. The method of claim 1, wherein the cells are hybridoma or CHO cells.

4. A method for reducing lactate production during cultivation of CHO, myeloma, or hybridoma cells, comprising cultivating CHO, myeloma, or hybridoma cells in culture medium in the presence of citric acid or citrate wherein said citric acid or citrate is maintained at a concentration of about 1 to 50 mmol/l during cultivation and wherein said citric acid or citrate is not bound in a chelate complex with iron or another transition metal ion.

5. The method of claim 4, wherein the cells are myeloma cells.

6. The method according to claim 4, wherein the cells are hybridoma or CHO cells.

* * * * *

EXHIBIT AA

US008460895B2

(12) **United States Patent**
Eisenkraetzer et al.

(10) **Patent No.: US 8,460,895 B2**
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(54) **METHOD FOR PRODUCING RECOMBINANT PROTEINS WITH A CONSTANT CONTENT OF PCO_2 IN THE MEDIUM**

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§ 371 (c)(1),
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C12P 21/06 (2006.01)

(52) **U.S. Cl.**
USPC **435/69.1**

(58) **Field of Classification Search**
None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,706,524 B2 * 3/2004 Wagner et al. 435/325
2002/0052046 A1 5/2002 Wagner et al.

FOREIGN PATENT DOCUMENTS

WO WO 2005/035748 4/2005

OTHER PUBLICATIONS

Bollati-Fogolin, "Expression of yeast pyruvate carboxylase in hGM-CSF-producing cho cells," Animal Cell Technology: From Target to Market, Proceedings of the 17th ESACT Meeting, pp. 241-243 (2001).

Bollati-Fogolin et al., "Impact of temperature reduction and expression of yeast pyruvate carboxylase on hGM-CSF-producing CHO cells," Journal of Biotechnology, vol. 109, No. 1-2, pp. 179-191 (2004).

Dezengotita et al., "Effects of CO2 and osmolality on hybridoma cells: Growth, metabolism and monoclonal antibody production," Cytotechnology, vol. 28, No. 1-3, pp. 213-227 (1998).

Kim et al., "Functional expression of human pyruvate carboxylase for reduced lactic acid formation of Chinese hamster ovary cells," Applied Microbiology and Biotechnology, vol. 76, No. 3, pp. 659-665 (2007).

Kimura et al., "Effects of elevated pCO2 and/or osmolality on the growth and recombinant tPA production of CHO cells," Biotechnology and Bioengineering, vol. 52, No. 1, pp. 152-160 (1996).

Pattison et al., "Measurement and Control of Dissolved Carbon Dioxide in Mammalian Cell Culture Processes Using an in Situ Fiber Optic Chemical Sensor," Biotechnology Progress, American Institute of Chemical Engineers, vol. 16, pp. 769-774 (2000).

Takuma et al., "Dependence on glucose limitation of the pCO2 influences on CHO cell growth, metabolism and IgG production," Biotechnology and Engineering, vol. 97, No. 6, pp. 1479-1488 (2007).

Zhu et al., "Effects of elevated pCO2 and osmolality on growth of CHO cells and production of antibody-fusion protein B1: a case study," Biotechnology and Progress 2005, vol. 21, No. 1, pp. 70-77 (2005).

* cited by examiner

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(57) **ABSTRACT**

A method for the recombinant production of a polypeptide in a eukaryotic host cell modified in the citrate cycle is described, wherein the method comprises cultivating the eukaryotic host cell in a suitable medium under conditions which allow the expression of the polypeptide, wherein the content of dissolved CO_2 (pCO_2) in the medium is maintained at a constant value in the range of 10% to 20%.

13 Claims, 36 Drawing Sheets

METHOD FOR PRODUCING RECOMBINANT PROTEINS WITH A CONSTANT CONTENT OF PCO_2 IN THE MEDIUM

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is the National Stage of International Application No. PCT/EP2009/001742, filed Mar. 11, 2009, which claims benefit to German Application No. 10 2008 013 899.1 filed on Mar. 12, 2008. The contents of the applications cited above are incorporated by reference in their entirety.

FIELD OF THE INVENTION

Background of the Invention

The present invention relates to a method for the recombinant production of a polypeptide in a eukaryotic host cell modified in the citrate cycle, wherein the cell is cultured in a medium with a content of dissolved CO_2 (pCO_2) which is maintained at a constant value in the range of 10% to 20%.

The ton-scale production of therapeutic proteins for specific therapies implies new requirements regarding expression and production systems. The main expression systems used are, amongst others, animal cell culture systems. The capability of animal cells to correctly fold and post-translationally modify proteins is, above all, a requirement for clinical application in humans. At present, almost 70% of all recombinant proteins are produced in animal cells in the pharmaceutical industry, most of these in CHO cells (*Chinese Hamster Ovary cells*) (Wurm, 2004). In comparison to microbiological systems, however, animal cell culture processes are characterized by longer generation time and lower final cell density in fermentations. Thus, product titres and space-time yields are lower than in microbiological processes. One possibility to compensate this disadvantage is metabolic engineering, i.e. controlling cell growth and minimizing apoptosis, programmed cell death, by genetic modification of the producing cells. In addition to genetic approaches, culture control strategies prove to be suitable optimization approaches the potential of which is often underestimated. It is, for example, possible to efficiently influence glycosylation, carbon metabolism, cell growth and cell death by using procedural monitoring and control strategies as well as the composition of culture media.

Thus, procedural development aims not only at the development of metabolically optimized cell lines but also at maximum exploitation of the potential of an existing cell line by optimum medium conditions and optimum process control. Apart from process parameters easily controllable such as oxygen content and temperature, more complex influence factors such as the content of dissolved carbon dioxide are taken into consideration for process control. In animal cell culture processes, CO_2 accumulates as final product in physically dissolved form and chemically dissociated as hydrogencarbonate in aqueous culture media. Today, the development towards high cell density processes using animal cells in fed batch processes results, in conjunction with industrially used large-volume fermenters and hydrostatic pressures prevailing therein, i.e. in CO_2 partial pressures of 150-200 mm Hg which, consequently, are five times higher than the cell physiological values of 31-54 mm Hg. For example, for the provision of polypeptides (cytokines) produced by recombinant CHO cells, said cells were cultivated at 37° C. and 5% CO_2 (36 mm Hg pCO_2) in order to express the recombinant cytokines (U.S. Pat. No. 6,406,888B1). Fogolin et al., Journal of Biotechnology, 2004, 109, 179-191 shows that the genetic modified cell line CHO-K1-hGM-CSF expresses a recombinant yeast pyruvate carboxylase (PYC2) at 37° C. and 5% CO_2. The results of this study revealed that the expression of PYC2 and a reduced culture temperature have an additive effect on the cell specific productivity of the genetic modified cell line CHO-K1-hGM-CSF. Moreover, the effects of elevated pCO_2, osmolarity on the growth rate and specific human tPA production rate of a recombinant CHO cell line have been studied by Kimura and Miller, Biotechnology and Bioengineering, 1996, 52, 152-160. The media used for the experiments in this study contain 36 mm Hg pCO_2 (5% CO_2), 140, 195, 250 mm Hg pCO_2. However, these authors were of the opinion that the highest recombinant protein production rate can be achieved at 37° C. and 36 mm Hg pCO_2 (5% CO_2). Adverse effects on growth and productivity for hybridomas, NS0, CHO, BHK and insect cells have been reported for such high pCO_2 concentrations. In industrial large-scale fermenters, the accumulation of CO_2 is a restrictive factor. The desorption of dissolved CO_2 from the culture medium is a challenge for process engineering. Thus, the oxygen supply for the cultured cells has to be provided. Correcting variables for enhancing the oxygen transfer into the liquid phase are, for example, stirrer velocity and volumetric gas flow. However, these cannot be freely modified due to the partially cell disrupting shear stress and foaming.

BRIEF SUMMARY OF THE INVENTION

Thus, the technical problem underlying the present invention is to provide an optimized method for the recombinant production of proteins in eukaryotic host cells which overcomes the disadvantages described above, i.e. which throughout provides an optimum content of physically dissolved CO_2 in the medium.

The solution of this technical problem is provided by the embodiments characterized in the patent claims. The strategy leading to the present invention was based on the approach of controlling the set value of pCO_2 with simultaneous control of dissolved oxygen, pH value and overpressure in the reactor. This rational approach of the decoupled control of as many parameters as possible associated with pCO_2-related difficulties resulted in the surprising finding that the yield of recombinantly produced protein unexpectedly increased with a pCO_2 value maintained constant in the range of 10% to 20% of dissolved CO_2. Moreover, the control of the pCO_2 concentrations over the entire process of fermentation had a positive effect on culture viability and allowed for a prolonged stationary phase of high cell density. The respective results of the strategy resulting in the present invention when applied to the culture of animal cells are summarized below:

(a) pCO_2 Control

Based on an in situ sterilisable pCO_2 probe, a pCO_2 controller was developed for the studies which would meet industrial requirements and which was implemented successfully in development fermentation. With simultaneous, independent control of pO_2 and pH, this pCO_2 controller allows both, pCO_2-static culture and the generation of set value profiles for pCO_2. Applications in fed-batch mode and chemostat mode were established successfully on the 1 L and 10 L scale. The control range is 1.5-25.0% pCO_2. Thus, for the parameter CO_2, it is possible to deal with problems in industry on a small scale.

(b) Overpressure Control

An overpressure valve was developed for scales 1 L (up to 150 mbar overpressure) and 10 L (up to 1000 bar overpressure) which may be used independently from the controls for

pCO_2, pO_2 and pH. Thus, problems of scale transmission in industrial bioprocesses (hydrostatic pressure, mixing time) can be represented on a small scale.

(c) pH Adjustment Agents

It was possible to show that the use of Na_2CO_3 for basic pH-adjustment results in increased viable cell density, prolonged viable culture period and, consequently, an increased space-time yield of a monoclonal antibody fusion protein in CHO cultures.

(d) Pressure-Controlled Sampling

The pressure-controlled sampling system allows the treatment of fermentation samples under bioreactor conditions (temperature, overpressure, pCO_2). For the first time, it is possible to study intracellular processes (e.g. intracellular pH) under bioprocess conditions.

(e) Effect of pCO_2 on the Intracellular pH Value of CHO Cells

The developed pCO_2 controller in combination with the pressure-controlled sampling allowed, for the first time, to observe the two-phase reaction of the intracellular pH-value with respect to changes in the pCO_2-value in the culture medium. Due to the dissociation equilibrium of CO_2 in aqueous solutions, the "chemical effect" as a direct effect causes a temporary change of the intracellular value (acidification of cytosol upon pCO_2 increase, alkalisation of cytosol upon pCO_2 neutralization). Contrary to this effect, there is a long-term and permanent reaction of the cell, the "physiological effect". For the first time, it was possible to observe this super-compensation by the deflection of the intracellular pH caused by the "chemical effect" in vitro and in situ (chemostat). The higher the pCO_2 gradient, the greater was the change of the intracellular pH.

(f) Findings Resulting from pCO_2-Controlled Bioprocesses with Recombinant CHO Cell Lines

- Static pCO_2 set value control increases process robustness.
- Set value profiles of pCO_2 may be used in combination with cell physiology studies under bioprocess for a rational development of bioprocesses.
- The pCO_2 levels are related directly to the intracellular pH value.
- The intracellular pH value is related to cell cycle phase distribution.
- An increasing gradient between the extracellular pH value and the intracellular pH value promotes formation and export of lactate.
- Apart from the pH control of the culture medium, a pCO_2 control in pH-static bioprocesses may influence the lactate formation via the intracellular pH value, even in glucose-limited process conditions.
- The recombinant cell line CHO-hGM-CSF-PYC2, which expresses the cytosolic pyruvate carboxylase, exhibited a pCO_2-sensitive energy metabolism. By increasing the controlled static pCO_2 level, it was possible to intensify the oxidative metabolism via an increase in the intracellular pH value in the course of culture. Furthermore, together with an increase in the statically controlled pCO_2 level, a prolonged viable culture period, efficient maintenance of the cell cycle in the G1 G0-phase, increased cell-specific productivity and an increase in the space-time yield by 100% was observed.
- In statically controlled pCO_2 bioprocesses, a prolonged culture period has to be attributed, primarily, to the increased pCO_2 level, not to osmolality.
- The optimum process values for controlled pCO_2 levels are found at 15% pCO_2.
- The control of pCO_2 and, thus, the inhibition of the accumulation of CO_2 in the aqueous medium of bioprocesses allows, also in HCO_3^--buffered media, a reliable online-determination of the respiratory quotient RQ in cell culture processes with exhaust gas analysis.

DESCRIPTION OF THE FIGURES

FIG. **1**: Schematic control loop of the cascaded pCO_2 controller developed

Undercut of the set value of pCO_2: PID 1 controls the opening width of the CO_2-MFC (0-5 $L \cdot h^{-1}$) installed in ratio-mode until the set value is reached. Exceedance of the set value for pCO_2: PID 1 initially closes CO_2-MFC and, upon continuous positive deviation of the set value, opens an additional N_2-MFC (0-15 $L \cdot h^{-1}$) via PID 2 (controller cascade).

FIG. **2**: Controlled gas concentrations of dissolved pO_2 and pCO_2 by ratio gassing and maximized CO_2-discharge due to increased gassing ratio in the 10 L stirring vessel reactor

50 mbar overpressure, 0.15 M NaCl, 37° C., 200 rpm, pH uncontrolled.

FIG. **3**: Controlled gas concentrations of dissolved pO_2 and pCO_2 by ratio gassing and maximized CO_2-discharge due to increased gassing ratio in the 10 L stirring vessel reactor at 750 mbar overpressure

0.15 M NaCl, 37° C., 200 rpm, pH uncontrolled.

FIG. **4**: Process scheme of the controlled overpressure system implemented in Biostat ES

FIG. **5**: Dynamic set value control of the control factors concentration of dissolved carbon dioxide pCO_2 (continuous line) and overpressure p (interrupted line) in the stirring vessel reactor

(Biostat ES, B. Braun International; industrial production medium, standard fermentation parameters) by developed PID controller in process control software LabView.

FIG. **6**: Titration profile of the pH control to pH 7.2 with 1 M NaOH upon a set value surge from 5% pCO_2 to 15% pCO_2

Biostat ES, ratio gassing 30 $L \cdot h^{-1}$, 10 L working volume, aqueous buffer solution with $NaHCO_3/NaH_2PO_4/Na_2HPO_4$ analogous to industrial production medium, 37° C., 750 mbar overpressure.

FIG. **7**: Titration profile of the pH control to pH 7.2 with 1 M Na_2CO_3 upon a set value surge from 5% pCO_2 to 15% pCO_2

Biostat ES, ratio gassing 30 $L \cdot h^{-1}$, 10 L working volume, aqueous buffer solution with $NaHCO_3/NaH_2PO_4/Na_2HPO_4$ analogous to industrial production medium, 37° C., 750 mbar overpressure.

FIG. **8**: Cumulative entry of the 1 M bases for pH control upon set value surge from 5% pCO_2 to 15% pCO_2

Biostat ES, ratio gassing 30 $L \cdot h^{-1}$, 10 L working volume, aqueous buffer solution with $NaHCO_3/NaH_2PO_4/Na_2HPO_4$ analogous to industrial production medium, 37° C., 750 mbar overpressure.

FIG. **9**: Fed-batch fermentation with constant 5% (v/v) of CO_2 in the gassing mixture and pH-adjustment agent NaOH and Na_2CO_3, respectively

Viable cell densities ZDL, viabilities VIA, product titre PRO (1 L, CHO-MUC1, 150 mbar overpressure, pH 7.2, 200 rpm, membrane gassing).

FIG. **10**: Fed-batch fermentation with variable CO_2 ratios in the gassing mixture and pH adjustment agent NaOH and Na_2CO_3, respectively

Viable cell densities ZDL, viabilities VIA, product titre PRO (1 L, CHO-MUC1, 150 mbar overpressure, pH 7.2, 200 rpm, membrane gassing; cf. also FIG. **11**).

FIG. **11**: Glucose and lactate concentrations in fed-batch cultivation of cell line CHO-MUC1 with pH-dependent variable CO_2 ratios in the gassing mixture

1 L, CHO-MUC1, 150 mbar overpressure, pH 7.2, 200 rpm, membrane gassing; cf. also FIG. **10**.

FIG. **12**: Osmolalities OSM and viable cell densities ZDL of a fed-batch fermentation

1 L, CHO-MUC1, 150 mbar overpressure, pH 7.2, 200 rpm, membrane gassing.

FIG. **13**: Combined parameters for the characterization of the different sampling methods in Biostat ES (10 L)

open, closed storage: reaction tubes completely filled with medium (50 mL, Falcon); sampling apparatus developed: gas-tight and closed syringe without overpressure completely filled with medium.

FIG. **14**: CO_2 loss with different sampling types under atmospheric pressure in Biostat ES

(0.15 M NaCl, 630 mbar overpressure), in situ and off-line CO_2 measurement YSI and AVL Compact 3; reaction tubes (50 ml, Falcon) completely filled with medium.

FIG. **15**: In situ CO_2 equilibrium concentrations (Biostat ES) for different overpressures (A), time-dependent CO_2 concentrations in the sample subsequent to collection by means of gas-tight syringe and subsequent storage without overpressure (B)

0.15 M NaCl, 37° C., otherwise standard fermentation conditions in the stirring vessel.

FIG. **16**: Photo of the pressure-controlled sampling unit of Biostat ES with reference to the components described in the text

FIG. **17**: Transparent support unit (**1**) of the pressure-controlled sampling apparatus developed with three different syringes (**2**-**4**) for different sample volumes and adapter sleeve (**5**) for precise insertion of the syringes into the support

Sample volumes can be pre-selected by locating the posterior stop plate (**6**) in position.

FIG. **18**: Top view of the support unit of the developed pressure-maintaining sampling apparatus with installed syringe

FIG. **19**: Comparison of the in situ CO_2 concentrations (in situ YSI; off-line AVL Compact 3) in the cell-containing production medium at the end of a fermentation of the CHO-MUC1 cell line at 750 mbar overpressure

Storage of the samples at 37° C. and without agitating during the measurement period; reaction tubes (50 mL, Falcon) completely filled with medium.

FIG. **20**: Flow cytometric pH_i measurements after 25 min (A,B) and 50 min (C), respectively, of pressureless incubation with the fluorescence dye SNARF-1; fluorescences (FL2, FL3) and their intensity ratio (ratio), respectively

Explanations: see text.

FIG. **21**: Flow cytometric pH_i measurements after 25 min isobaric incubation in the developed pressure-controlled sampling apparatus with the fluorescence dye SNARF-1 (D) and after isobaric storage (25 min) and subsequent pressureless incubation (25 min) with the fluorescence dye SNARF-1 (E); fluorescences (FL2, FL3) and their intensity ratio (ratio), respectively

Explanations: see text.

FIG. **22**: Temporary intracellular alkalizing effect of different pCO_2 depletions in the medium on the CHO-MUC1 cell line cultured in the medium

pCO_2 depletion by gassing the SNARF-1-dyed cell suspension in vitro with air at different incubation time. For all samples, the total duration of incubation prior to flow cytometric evaluation of intracellular pH is identical with the duration of neutralization by gassing with air.

FIG. **23**: Initial intracellular alkalizing effect (<5 min) upon depletion of dissolved CO_2 from the culture medium on the CHO-MUC1 cell line cultured therein and subsequent acidification of cytosol due to physiological effect (<40 min)

CO2 depletion by gassing the SNARF-1-dyed cell suspension in vitro with air at different incubation stages. For all samples, the total duration of incubation prior to flow cytometric evaluation of intracellular pH is identical with the duration of neutralization by gassing with air.

FIG. **24**: Biphasic pH_i curves at different start pCO_2 concentrations of 5%, 7% and 10%. Initial intracellular alkalizing effect upon depletion of dissolved CO_2 from the culture medium on the CHO-MUC1 cell line (chemical effect) and subsequent supercompensation of the alkalization due to the physiological effect below initial pH_i

CO2 depletion by gassing the SNARF-1-dyed cell suspension in vitro with air at different incubation time. For all samples, the total duration of incubation prior to flow cytometric evaluation of intracellular pH is identical with the duration of neutralization by gassing with air.

FIG. **25**: Continuous pCO_2-controlled process (1.0 L, D=0.8 d^{-1}, pH 7.0, adjustment agent Na_2CO_2 surges of 2.5% to 5.0% and from 5.0% to 10%, respectively

FIG. **26**: Viable cell numbers ZDL and viabilities VIA at 5%, 15%, 25% and 5-15% pCO_2, respectively

CHO-MUC1, 10 L fed batch, 750 mbar overpressure, standard fermentation conditions.

FIG. **27**: G0G1 phase fraction of the cell cycle distributions in processes with different pCO_2 set value controls

CHO-MUC1, 10 L fed batch, 750 mbar overpressure, standard fermentation conditions.

FIG. **28**: Turning point in the cell cycle phase fraction G0G1, corresponding pCO_2 value and intracellular pH_i-value in the industrial pCO_2 profile

5-15%, CHO-MUC1, 10 L fed batch, 750 mbar overpressure, standard fermentation conditions.

FIG. **29**: G0G1 phase fraction and intracellular pH-value pH_i at 25% pCO_2

CHO-MUC1, 10 L fed batch, 750 mbar overpressure, standard fermentation conditions.

FIG. **30**: Specific rates of glutamine consumption qGLN for different pCO_2 set value profiles

CHO-MUC1, 10 L fed batch, 750 mbar overpressure, standard fermentation conditions.

FIG. **31**: Specific yield coefficients Y related to glutamate GLT per glutamine GLN

CHO-MUC1, 10 L fed batch, 750 mbar overpressure, standard fermentation conditions.

FIG. **32**: Cell-specific lactate formation rates qLAC for different pCO_2 set value profiles

CHO-MUC1, 10 L fed batch, 750 mbar overpressure, standard fermentation conditions.

FIG. **33**: Lactate formation LAC for different pCO_2 set value profiles

CHO-MUC1, 10 L fed batch, 750 mbar overpressure, standard fermentation conditions.

FIG. **34**: Product titre PRO MUC1-IqG

10 L fed batch, 750 mbar overpressure, standard fermentation conditions.

FIG. **35**: Glucose-limited chemostat process (1 L) of cell line CHO-MUC1

Cell-specific lactate formation qLAC increased by pH control and increase in pCO_2 set value, respectively; specific growth rate μ, flow rate D.

FIG. **36**: Glucose-limited chemostat process (1 L) of cell line CHO-MUC1

Curves of glucose, lactate and glutamine concentration, respectively (GLC, LAC and GLN).

FIG. **37**: (a) conventional metabolic pathway of the substrate glucose via pyruvate to lactate; (b) enabling the transformation of pyruvate and hydrogen carbonate into oxaloacetate and transfer of subsequent malate into the tricarbonic acid cycle (TCA) by cytosolic pyruvate carboxylase PYC2; modified according to Irani (1999)

FIG. **38**: Fed-batch cultivation of cell line CHO-hGM-CSF-PYC2: curve of viable cell densities (filled symbol) and pCO_2 concentrations (open symbol), adjustment of pCO_2 to 5% and 15% and/or no adjustment of pCO_2, respectively

FIG. **39**: Viable cell densities ZDL and viabilities VIA at uncontrolled and controlled pCO2 concentrations of 5% and 15%, respectively, in 1 L fed-batch of cell line CHO-hGM-CSF-PYC2: pCO_2 control allows prolonged production period with highly viable cells

FIG. **40**: Osmolality OSM and cumulative lye inlet (1 M Na_2CO_3) with uncontrolled pCO_2, controlled pCO_2 at 5% and 15%, respectively

Enhanced lye inlet during the exponential growth phase with uncontrolled pCO_2 in comparison to pCO_2-controlled processes; the osmolality curve is similar in all processes.

FIG. **41**: Lactate concentrations LAC in the individual bioprocesses

pCO_2 control prevents rapid and high lactate formation.

FIG. **42**: Cell-specific lactate formation rates qLAC

Lactate metabolism with uncontrolled pCO_2 ($\geqq$180 h) and controlled pCO_2 of 5% ($\geqq$220 h) can be observed; no metabolism of lactate with controlled pCO_2 of 15%.

FIG. **43**: Cell-specific product formation rates SPR of the recombinant growth factor hGM-CSF with different pCO_2 process conditions

Inverse activity in pCO_2-uncontrolled process in comparison to pCO_2-controlled processes: decrease of SPR upon entry into stationary growth phase without pCO_2 control; in contrast, high increase in SPR with controlled pCO_2 of 5% and 15%, respectively.

FIG. **44**: Absolute final concentration of growth factor hGM-CSF produced at the end of fermentation (80% viability of the culture) for the relevant pCO_2 profile

FIG. **45**: Cell cycle distribution ratio S/(G0G1), cell-specific product formation rate SPR and intracellular pH value pH_i in the course of a pCO_2-uncontrolled-uncontrolled fed-batch culture of cell line CHO-hGM-CSF-PYC2

Decrease of pH_i of about 0.3 subsequent to the beginning of feed, increase in pH_i at entry into the growth phase.

FIG. **46**: Cell cycle distribution ratio S/(G0G1), cell-specific product formation rate SPR and intracellular pH value pH_i in the course of a fed-batch culture of cell line CH-hGM-CSF-PYC2 CO_2-controlled to 5%

Decrease of pH_i of about 0.4 subsequent to the beginning of feed, increase in pH_i at entry into the stationary growth phase.

FIG. **47**: Cell cycle distribution ratio S/(G0G1), cell-specific product formation rate SPR and intracellular pH value pH_i in the course of a fed-batch culture of cell line CH-hGM-CSF-PYC2 pCO_2-controlled to 15%

No decrease of pH_i subsequent to the beginning of feed, initially minor reduction of the pH_i increase at entry into the stationary growth phase, subsequently increase with the SPR.

FIG. **48**: Cell-specific productivities and cell cycle phase fractions G0G1 of the fed-batch processes with uncontrolled and/or up to 5% and 15% pCO_2 controlled pCO_2

1 L scale, cell line CHO-hGM-CSF-PYC2, standard fermentation conditions.

FIG. **49**: OUR, CPR and RQ of the fed-batch process with controlled pCO_2 of 5%

CHO-hGM-CSF-PYC2, 1 L fed batch, standard fermentation conditions.

FIG. **50**: OUR, CPR and RQ of the fed-batch process with controlled pCO_2 of 15%

CHO-hGM-CSF-PYC2, 1 L fed batch, standard fermentation conditions.

FIG. **51** shows the removal of flanking yeast-specific sequences from the 5'- and 3'-regions of the PYC2 gene.

DETAILED DESCRIPTION OF THE INVENTION

Thus, the present invention relates to a method for the recombinant production of a polypeptide in a eukaryotic host cell modified in the citrate cycle, wherein the method comprises the following steps:

(a) cultivating the eukaryotic host cell in a suitable medium under conditions which allow the expression of the polypeptide, wherein the content of dissolved CO_2 (pCO_2) in the medium is maintained at a constant value in the range of 10% to 20%; and

(b) recovering the polypeptide from the cell or from the medium.

Due to the method of the present invention, a very high product concentration is achieved, resulting in a reduction of recovery costs. Thus, the increase in the product titre allows the production of a desired amount of products in a small culture volume, which results in lower investment costs.

The person skilled in the art is familiar with genes the products of which are involved in the citrate cycle and, thus, he is able to produce eukaryotic host cells which are modified in the citrate cycle and which are suitable for the method of the present invention (cf., e.g., Irani et al., (1999), Chen et al., (2001); Paredes et al., (1999), Bell et al., (1995)). In addition, the person skilled in the art can use host cells exhibiting modifications as the above which are already obtainable from sources accessible to the public. Furthermore, the person skilled in the art is also familiar with suitable culture conditions and media for the cultivation of such cells as well as with conditions under which the gene expressing the desired recombinant protein is expressed to a high degree. In order to achieve a constant content of dissolved CO_2 in the medium, the person skilled in the art can preferably make use of the control units which are described in the following Examples.

The term "constant value" or "stable value" as used herein refers to the fact that the content of dissolved CO_2 does not deviate more than 20% from the desired or programmed set value, i.e., for example, it is in the range of 8-12% for a set value of 10%.

The method of the present invention can be carried out using different eukaryotic production cell lines. The modification in the citrate cycle, preferably a genetic modification, can be effected, for example, by insertion of additional genes of the same organism or of another organism into the DNA or by a vector or by enhancing or attenuating the activity or expression of a gene by introducing a more effective promoter, e.g. from CMV, or by corresponding mutations in the coding region of the gene which lead to the substitution, deletion or addition of one or more amino acid residues.

In a preferred embodiment of the method of the present invention, the host cell is an animal cell, preferably a mammalian cell or insect cell. Such cells, in particular cells for the efficient production of recombinant polypeptides are known to the person skilled in the art. The following cell lines can be indicated by way of example: mammalian cells such as CHO cell lines, e.g. CHO-K1, BHK, such as BHK-21, hybridoma, NS/0, other myeloma cells and insect cells or other higher cells. The use of cells which do not produce in a growth-dependent manner is particularly preferred.

In a further embodiment of the method of the present invention, the host cell is a host cell that is modified in the citrate cycle. The different metabolic pathways of the citrate cycle have been known for a long time, as have the genes involved and their control. Thus, the person skilled in the art is able to carry out the desired modification(s) according to standard methods. A particularly preferred host cell modified in the citrate cycle is a cell expressing cytosolic pyruvate carboxylase, preferably a cytosolic pyruvate carboxylase from *Saccharomyces cerevisiae* (PYC2, isoenzyme 2). The gene coding this carboxylase and suitable expression vectors for the transformation of eukaryotic cells are described, amongst others, in U.S. Pat. No. 6,706,524 and Stucka et al. (1991); see also Wagner (1998); Irani (1999); Bollati Fogolin (2003); WO 00/46378.

The cell line CHO-hGM-CSF-PYC2, which contains the plasmid pCMVSHE-PYC2, the production of which plasmid is also described in U.S. Pat. No. 6,706,524 in detail, is particularly preferred for the method of the present invention.

The method of the present invention allows to obtain high yields of polypeptides, such as glycoproteins, fusion proteins, antibodies and their fragments, interferons, cytokins, preferably hGM-CSF, growth factors, e.g. erythropoietin (EPO), hormones etc. in a recombinant manner.

An essential feature of the method of the present invention is the fact that the content of dissolved CO_2 is maintained constant using suitable measures as described in the following Examples. The person skilled in the art can—dependent on the cell line used—determine the particularly suitable content of CO_2 within the range of 10% to 20% by means of standard assays. The content of dissolved CO_2 (pCO_2), which is to be maintained constant, is preferably in the range of 10% to 20%, more preferably in the range of 11% to 19%, more preferably in the range of 12% to 18%, more preferably in the range of 12.5% to 17.5%, more preferably in the range of 13% to 17%, more preferably in the range of 14% to 16%.

The method of the present invention can be carried out using known methodologies, e.g. batch, fed-batch, chemostatic process or perfusion culture, with the fed-batch process being preferred. All established types of culture vessels, such as stirring vessels, can be used for these processes. Preferably, the culture system should allow for high cell densities.

In principle, any medium suitable for the cultivation of eukaryotic cells can be used as culture medium. For the cultivation of mammalian cells, it is possible to use media which are based on the known formulations, such as IMDM, DMEM or Ham's F12, and which possibly have been optimized for the method of the present invention so that there is no limitation with respect to specific individual components. This can be achieved, for example, by a higher concentration of individual components. In principle, it is also possible to dose single nutrients separately from the medium if necessary.

The pH range is preferably between 6.7-7.7, particularly preferred between 7-7.3. However, other pH ranges are also conceivable. The temperature range is preferably between 35° C.-38.5° C., particularly preferred at 37° C., e.g., for mammalian cells, such as CHO cells. However, other temperature ranges are also conceivable, such as e.g. <35° C. in the case of non-mammalian cells.

In order to maintain the content of dissolved CO_2 in the medium constant, it is preferably maintained constant by using a control system with a cascaded pCO_2 controller via mass flow controllers (MFC), for example, as described in the Examples below. An approach, wherein, via the MFC (a), the CO_2 ratio in the supply air is increased by an overcut of the set value of pCO_2 in the medium, (b) the CO_2 ratio in the supply air is decreased by an exceedance of the set value of pCO_2 in the medium, and (c) the cascaded controller opens an additional MFC for the delivery of N_2, preferably in combination with step (b), if the exceedance of the set value cannot be sufficiently compensated by a decrease of the CO_2 ratio in the supply air, is particularly preferred and advantageous.

Example 1

Material and Methods

(A) Cell Lines

All cell lines used were adapted to culture in suspension in serum-free media.

(1) CHO-MUC1

The cell line CHO-MUC1 used in the present invention is based on a CHO-K1 cell line of ATCC (American Type Culture Collection, Rockville, USA) (CCL-61). It was transfected with the recombinant plasmid MUC1-IgG2a-pcDNA3 (Link et al., 2004) in confluent culture using electroporation. Apart from resistance against the antibiotic geneticin (G418), the construct, which is under constitutive control of the cytomegalovirus promoter (CMV), also mediates the expression of the MUC1-IgG2a fusion protein. The extracellular part of the human breast cancer-associated mucin glycoprotein MUC1 (C-terminal) is fused (N-terminally) to the Fc part of a murine immunoglobulin of subclass 2a (IgG2a) via an enterokinase cleavage site and, thus, it is secreted into the medium. Selection and subsequent subcloning resulted in clone CHO-MUC1-IgG2a-PH3931-16TR which was used in the present invention (Link, 2003). This cell line exhibits genicitin resistance up to a concentration of 400 $\mu g\ mL^{-1}$.

(2) CHO-hGM-CSF-PYC2

Clones of the recombinant CHO-hGM-CSF-PYC2 cell lines were provided by courtesy of M. Bollati Fogolin (GBF mbH, Braunschweig). They were generated by co-transfection of the recombinant cell line CHO-K1-hGM-CSF (Bollati Fogolin, 2001) with plasmids pCMVSHE-PYC2, which contains the gene coding cytosolic pyruvate carboxylase from yeast (Wagner, 1998; Irani, 1999) and pHMR272, which codes hygromycin resistance.

The plasmid pCMVSHE-PYC2 was generated as follows. The cDNA of pyruvate carboxylase (PYC2, isoenzyme 2) from *Saccharomyces cerevisiae* was obtained from R. Stucka (Stucka et al., 1991). In order to avoid possible interactions of the yeast vector fragment bordering the coding region, the cDNA was trimmed down to the essential coding sequences by endonuclease restriction and ligation with small PCR fragments according to a procedure well known in the art (FIG. **51**).

The modified cDNA was placed under the transcription control of a CMV promoter. The resulting pCMVPYC2 plasmid also contains an ampicillin-resistance gene. It will be clear that other promoters and resistance genes or general selection markers can be used.

FIG. **51** shows the removal of flanking yeast-specific sequences from the 5'- and 3'-regions of the PYC2 gene.

First using PvuII, a 678 bp fragment was cut out from the 5'-end, the missing 381 bp were ligated by means of PCR (polymerase chain reaction) via specific primers, and a HindIII restriction site was built on before the coding sequence.

For the 3'-end, a corresponding procedure was carried out via the ClaI restriction site by removal of 469 bp and ligation with a fragment 176 bp long. The fragment was provided with a HindIII and SmaI restriction site at the 3'-end.

Clone CHO-hGM-CSF-PYC2-4D5 (Bollati Fogolin, 2003) was used for the studies carried out in the present invention. The cell line secretes the recombinant, glycosy-

lated "human granulocyte-macrophage colony-stimulating factor" (hGM-CSF) into the culture medium and exhibits hygromycin resistance up to a concentration of 250 μg mL^{-1}.

(3) CHO-hGM-CSF

The cell population that was derived by analogous transfection of the CHO-hGM-CSF cell line with plasmid pHMR272 alone and subsequent selection was used as reference for the PYC2 expressing clone. This mixed population of different individual clones exhibits hygromycin resistance up to a concentration of 250 μg mL^{-1} (Bollati Fogolin, 2003).

(B) Culture Media

For strain maintenance and selection, the above-indicated antibiotics were used in appropriate concentrations.

(1) ProCHO4-CDM

The serum and protein-free medium ProCHO4-CDM (Biowhittaker Europe) which is commercially available was used for the cultivation of the CHO-MUC1 cell line in strain maintenance and small scale fermenter system (1 L, Applikon) for preliminary studies of the effect of the pH tritration agents. The medium has a glucose content of 4.30 gL^{-1} and contains 5.00 gL^{-1} HEPES. It was supplemented with $NaHCO_3^-$ (final 3.78 gL^{-1}), glutamine (final 4.1 mM), hypoxanthine (final 0.1 mM) and thymidine (final 0.1 mM). The pH value was adjusted to pH 7.0.

(2) Industrial Production Medium (Roche Diagnostics GmbH)

This medium was prepared according to the instructions with ultrapure water (SQ, Millipore) and supplemented for all cell lines. All media were adjusted to pH 7.0 by titration with 4 M NaOH. Subsequent to sterile filtration (0.2 μm) and sterile test (72 h, RT), these serum-free media were stored for up to one month (4° C.). The glucose concentration of the main fermentation medium is 8.0 gL^{-1}. For the feeding method, a medium concentrate with high glucose concentration was used.

(C) Off-Line Analysis

(1) Assessment of Cell Number, Viability and Sterility

The sample which were collected daily from the appropriate suspension cultures were examined microscopically for contamination (bacteria, fungi). Cell number and viability were assessed manually (light microscopy) and automatically (ViCell XR, Beckman Coulter, Krefeld). The ratio of live cells was determined using the exclusion method with a 0.5% (w/v) trypan blue solution. In order to identify dead cells, 0.2% trypan blue solution was added to the cell suspension in a mixture ratio of 1:1, carefully mixed and the number of dyed cells was determined by counting eight large squares of a counting chamber according to Neubauer (depth 0.100 mm, haemacytometer) under a light microscope with 100-fold magnification. By subtracting the resulting concentration of dead cells from the concentration of the cells in total, the concentration of viable cells in suspension is determined. Their ratio with respect to the concentration of the cells in total is referred to as viability. This measuring principle was automated in ViCell XR. Mixing with trypan blue and the discrimination between live and dead cell is effected by means of the contrast ratio. To this aim, an integrated camera supplies the corresponding images for the computer-assisted analysis.

(2) Analysis of the Media

Substrate, metabolite and product concentrations contained in the medium are accessible from the cell-free medium supernatants (10 min., 200 g) of the culture using the methods described below. The temporary storage of the cell-free supernatants is carried out at −80° C. If not indicated otherwise, all measurements and calibrations are carried out according to the manufacturer's instructions.

Glucose

In order to evaluate the content of glucose in culture supernatants, an automatic glucose analyser (EBIO Compact, Eppendorf, Hamburg) was used. The measurement is based on a coupled enzymatic electrochemical process wherein the hydrogen peroxide which, in addition to gluconic acid, is released by the immobilized glucose oxidase is measured amperometrically at a Pt/AgCl/Ag electrode. The electrode current resulting from the oxidation of the hydrogen peroxide into oxygen is directly proportional to the hydrogen peroxide concentration and, thus, to the glucose concentration of the sample.

Lactate

Lactate is also quantified according to an electro-enzymatic principle in an analyser (Yellow Springs Instruments, Ohio, USA). By means of immobilized lactate oxidase, lactate is reacted into pyruvate and hydrogen peroxide. The latter can be quantified as in glucose quantification.

Glutamine and Glutamate

The simultaneous quantification of glutamine and glutamate is carried out according to the principle described for glucose quantification using two immobilized enzymes on different sensors. Glutamate oxidase catalyses the reaction of glutamate with oxygen into α-ketoglutarate and hydrogen peroxide with elimination of ammonia. At the glutamine sensor, glutaminase first effects the formation of glutamate which is then reacted in an analogous manner. Thus, the glutamine sensor also detects free glutamate in the solution which is factored in by the analyser.

Osmolality

Osmolality is a measure for the number of dissolved particles per measuring unit of the solvent. According to the definition, deionised water freezes at 0° C. under normal conditions. A related depression of the freezing point of 1.858 K corresponds to an osmolality of 1 osmol/kg. The measurement of osmolality using the freezing point osmometer starts with cooling the sample to −7.0° C. The crystallisation of the subcooled solution is induced by means of a seed crystal and the quantity of heat produced is measured until the freezing point is reached. This difference to the pure solvent water is directly proportional to the osmolality of the sample.

Amino Acids

The concentration of free amino acids in the culture supernatant was determined by reverse-phase HPLC (High Performance/Pressure Liquid Chromatography) (Büntemeyer, 1988). To carry out the analyses, a fully automated HPLC system D450 (Kontron Instruments, Echingen) with two high pressure pumps (Model 420), automatic sampling (Model 460) as well as a fluorescence detector (Model SFM 25) and computer-assisted evaluation unit (KromaSystem 2000, version 1.60) was used. Separation was carried out using a RP_{18}, column (Ultrasphere ODS, 150 mm×4.6 mm, particle size 5 μm, Beckman, Munich) with octadecylsilicate as stationary phase. To this, a further RP_{18} column (Hypersil ODS, 10 mm×4.6 mm, particle size 10 μm, Techlab, Erkerode) was proposed in order to increase the dwell time. Prior to separation, the derivatization of the amino acids by ortho-phthaldialdehyde (OPA) (Sigma, Deisenhofen) (Lim, 1987; Büntemeyer, 1991) occurred. The primary amino groups of the amino acids react in alkalic medium (pH>9) with OPA and 3-mercaptopropionic acid to fluorescent isoindol derivatives (FIG. **2.1**). Detection was carried out at an emission wavelength of 450 nm subsequent to excitation with light of a wavelength of 340 nm.

The cell-free culture supernatants (500 μl) were mixed with trichloroacetic acid (100 μl, 36% w/v) and the precipitated proteins were sedimented (10 minutes, 15000 g). The

intermediate phase was recovered (250 μl) and neutralized (12.5 μl NaOH 1 M). This stock solution was added to the derivatization in suitable pre-dilution and alkalization with sodium borate buffer (0.4 m, pH 9.5), wherein, due to the instability of the isoindol derivatives, the derivatization was carried out only immediately before HPLC separation by automatic addition of the derivatization reagent (50 mg OPA in 1 ml methanol, 100 μl 3-mercaptopropionic acid and 9 ml 0.6 M sodium borate buffer, pH 10.4) to the sample. The gradient used for the HPLC separation of the amino acid derivatives (Büntemeyer, 1991) was run from the more polar (99% v/v 0.1 m sodium acetate pH 7.5, 1% v/v tetrahydrofurane) to the more non-polar buffer (30% 0.085 M sodium acetate pH 5.2, 70% methanol) which allowed to quantify all amino acids with the exception of cysteine and proline. Proline as secondary amine exhibits no reaction and cysteine forms, together with OPA, a non-fluorescent derivative.

(3) MUC1-IgG2a ELISA

The MUC1-IgG2a antibody produced by the CHO-MUC1 cell line cultivated in this study is a representative of the murine immunoglobulin G (IgG) whose H chain is of type y and whose subclass is 2a. It is N-terminally linked to MUC1 whereby the detection of a molecule of this antibody always includes the detection of a molecule of fused MUC1. In order to prevent the detection of incompletely translated product, only cell viabilities of more than 80% were taken into consideration. The quantitative evaluation of MUC1-IgG2a in culture supernatant was carried out by means of ELISA.

The quantification of the MUC1-IgG fusion protein was carried out according to the studies by Link (2003) (Parker, 1990; Link, 2003). 96-well immunoplates (F96 Maxisorp, Nunc, Wiesbaden) were used as adsorbing solid phase for MUC1-IgG quantification by means of ELISA. The catching antibody adsorbed to the solid phase was a commercially available goat anti-mouse IgG (M-8642, Sigma, Deisenhofen) which was obtained by immunisation of goats against murine IgG. The supplied antibody (1 mg) was dissolved in PBS (25 ml, final 40 μg mL^{-1}), aliquoted (1 mL) and stored at −20° C. Prior to use, it was diluted in PBS to 3 μg mL^{-1}. The supernatants to be quantified as to MUC1-IgG2a as well as a MUC1-IgG2a standard were used as determinable antigens. The standard was pre-diluted to 1 μg mL^{-1} in dilution buffer prior to use in ELISA.

The alkaline phosphatase conjugate goat anti-mouse IgG AP of the catching antibody (A-5143, Sigma, Deisenhofen) which carries alkaline phosphatase (AP) as substrate-reacting enzyme and specifically binds to the heavy y chain of the murine IgG was used as enzyme-marked antibody. It was stored at 4° C. By the alkaline phosphatase, the substrate p-nitrophenol phosphate (pNPP, N-2770, Sigma, Deisenhofen) was reacted into yellow p-nitrophenol which was determined photometrically. The extinction of the resulting staining was evaluated using a spectrophotometric analyser (405 nm, Wallac Victor^2, Perkin Elmer Life Sciences, Bad Wildbach).

In order to coat the Maxisorp immunoplates with the catching antibody goat anti-mouse IgG, this was pipetted into each of the wells of the microtitre plate (100 μL per well). Subsequently, the closed test plate was incubated (4° C., 14 h) and after removing the buffer by rapidly turning the microtitre plate upside down and slapping it, it was rinsed with washing buffer (10 times) and tapped dry. In order to block the unspecific binding sites of the catching antibody, blocking buffer was added to each well (200 μL per well) and the covered plate was incubated (37° C., 2 h). The plate was then washed with washing buffer (3 times) and tapped to dry it off.

The cell-free culture samples to be analysed were first centrifuged (15000 g, 3 minutes) and the resulting supernatant was used as product-containing sample for further analysis. A suitable pre-dilution in dilution buffer was carried out in order to obtain comparable resulting intensities from standards and samples for the evaluation of the colour reaction, allowing conclusions with respect to product concentrations. BSA contained in the dilution buffer served as neutralizing agent for unspecific binding sites. For a double-determination of the samples (columns 3-12) and the standard (columns 1 and 2), respectively, 280 μL per well were pipetted into row H of pre-dilution plates (96 wells, Nunc, Wiesbaden) divided into A1 to H12. Subsequently, a 1:2 dilution was carried out step-by step by transferring 140 μL per step into the wells of the following row which each contained 140 μL of dilution buffer.

The wells of the coated test plate are loaded each with 100 μL of the pre-diluted samples and standards with the dilution increasing with each row in analogy to the pre-dilution plate and the covered plate was incubated (37° C., 1 h).

To balance the spectrometer against a blank sample, two wells (bottom right) were treated only with dilution buffer. Subsequently, after slapping it, the plate was rinsed with washing buffer (10 times) and the plate was tapped to dry it off. In the following step, the enzyme, labelled goat anti-mouse IgG AP antibody was first taken up into dilution buffer in the ratio 1:1000 and, subsequently, pipetted into the wells of the test plate (100 μL per well). Incubation was carried out anew (37° C., 1 h) and the test plate was then washed with washing buffer (10 times) after removing the residue buffer by slapping. Since substrate pNPP is sensitive to light, all following steps were carried out in quick succession in the dark. The reaction of p-nitrophenyl phosphate into yellow product by the antibody-bound alkaline phosphatase was induced by adding substrate to each well of the test plate (100 μL). The plate was incubated in darkness (room temperature, 10-30 minutes) until an intensive yellow staining (extinction of 0.7-0.8) was observed in the well with the reacted standard. The evaluation of the intensities of the staining was carried out by spectrophotometry at 405 nm.

(4) hGM-CSF EILSA

Competitive ELISA (Bollati Fogolin, 2002) was used to detect the cytokine hGM-CSF. 96-well immunoplates (F96 Maxisorp, Nunc, Wiesbaden) were used as adsorbing solid phase for quantification of hGM-CSF by ELISA. Recombinant, unglycosylated HGM-CSF from *E. coli* which is commercially available (Leucomax, active ingredient: molgramostim, Schering-Plough corporation Kenilworth, N.J., USA) was used for coating the Maxisorp plates and as concentration standard. A monoclonal mouse-anti-GM-CSF antibody (M7E10) (Etcheverrigaray, 1998), which was kindly provided by Ms. Bollati Fogolin, was used as primary antibody. This antibody is directed against an hGM-CSF domain which is accessible independently of the glycosylation status of the protein. The enzyme labelled antibody was a peroxidase conjugate goat-anti-mouse-IgG-HRP that carries horseradish peroxidase as substrate-reacting enzyme and specifically binds to the Fab_2 fragment of the murine IgG primary antibody (Dianova 115-035-072, 0.8 mg mL^{-1}). The substrate 1,2-phenylenediamine-dihydrochloride (OPD, Fluka) was reacted into an orange end product by horseradish peroxidase after the enzyme reaction was stopped. The extinction was evaluated by photometry. The extinction of the resulting staining was read in spectrophotometric analyser (492 nm, measuring time 1 second/well, Wallac Victor^2, Perkin Elmer Life Sciences, Bad Wildbach).

In order to coat the Maxisorp immunoplates with hGM-CSF from *E. coli* as competitor to glycosylated hGM-CSF from the fermentation supernatant, the first was pipetted into each well of the microtitre plate (per well 16.5 ng in 100 μL coating buffer). Subsequently, the closed test plate was incubated in an atmosphere saturated with water vapour (37° C., 1 h, subsequently 4° C., 14 h) and after removing the residue buffer by slapping rinsed with washing buffer (10 times) and tapped to dry off. In order to determine the standard curve, dilutions of the unglycosylated hGM-CSF standards in the range of 200-0.195 ng mL^{-1} were carried out in dilution buffer. The samples from the culture supernatants were also diluted sequentially 1:2 in dilution buffer and pipetted together with the prepared standards into the wells of the coated microtitre plate (100 μL per well). Plain dilution buffer is used as control for complete binding without competition of hGM-CSF-containing standard or culture supernatant. Then, the primary antibody M7E10 (1:100 000 in dilution buffer, 100 μL per well) was added to the wells of the plate loaded with samples and standards and it was incubated (37° C., 1 h). After removing the solution and washing the plate with washing buffer (7 times), an incubation was carried out (37° C., 1 h) with conjugate antibody (1:5000 in dilution buffer, 100 μL per well). After washing the plate (7 times), the enzyme reaction was started by adding the freshly prepared dye solution. After 15 minutes incubation, the reaction was stopped by adding stop solution (50 μL per well) and the resulting extinction is evaluated at a wavelength of 492 nm (per 1 s).

(5) Flow Cytometric Cell Analysis

The correspondingly prepared samples were measured using FACSCalibur Calibur flow cytometer (Becton Dickinson, San Jose, Calif., USA) and CellQuest software version 3.3 (both Becton Dickinson, San Jose, Calif., USA). Off-line analysis of the data was carried out using CellQuest 3.3, Modfit and FCSassist 1.0 on a G4 Apple Macintosh computer.

Cell Cycle Phase Distribution

To determine the individual cell phases (G1, S, G2, M), the culture sample in form of a single cell suspension was liberated from supernatant (200 g, 7 min) and washed twice in ice-cold PBS (200 g, 7 min). The resulting pellet was resuspended dropwise in a mixture (−20° C.) of 80% (v/v) methanol (high-grade, Merck) under intensive agitation and incubated (4° C., 2 h—2 months). The cells were then pelleted (200 g, 7 min), subsequently, they were resuspended in a mixture of 0.1% (w/v) saponine (Roth) in PBS and washed twice (200 g, 7 min). Resuspension of 4×10^6 cells in 1 mL dye solution (0.1% w/v saponine, 1 g mL^{-1} RNase S (Sigma), 0.02 mg mL^{-1} propidium iodide (Sigma)) and incubation (RT, 30 min) resulted in a pink-stained cell pellet. First the enzymatic degradation of the RNA was stopped by storage on ice and, then, the stained cells were subjected to flow cytometry. Measuring was carried out according to the manufacturer's instructions and protocols of the relevant literature regarding flow cytometry (Melamed, 1990; Givan, 1992; Shapiro, 1994; BD-Biosciences 2000).

Intracellular pH Value

The intracellular pH value (pH_i) of animal cells is related to different cellular functions. Modifications in cell growth, metabolism and protein production as well as in enzyme activities, transport mechanisms, proliferation induction and maintenance energy requirement involve variances in pH_i (Madshus, 1988; Grinstein, 1989). Thus, the control of pH_i is of fundamental biological significance for the cell. The isoelectric points of many biologically important macro-molecules in the cell are near the physiological value of pH 7 so that comparatively small modifications in proton concentration can have major effects on the conformation and interaction of proteins and nucleic acids. To prevent an acidification of cytosol, the H^+ protons are directly removed from the cell or hydrogen carbonate ions HCO_3^- are introduced into the cell in order to neutralize the protons in cytosol. For this pH_i control, different mechanisms are available in animal cells (Madshus, 1988; Reusch, 1995): Na^+/H^+ exchanger, Na^+ dependent and independent Cl^-/HCO_3^- antiporter, Na^+/HCO_3^- symporter, H^+ translocating ATPases (proton pumps).

In contrast to the external pH (pH_e) which, in animal cell cultures, is in the range of 6.6-7.4, the above-mentioned mechanisms allow that the cell can actively maintain internal pH values which deviate from the external values by 0.1-0.5 pH units. The cell organelles have distinctive pH values in order to fulfil their biological functions. Furthermore, sensitively controlled pH gradients are important for posttranslational protein sorting and processing. Dysfunctions of this intracellular control may occur in cell cultures due to accumulation of organic acids and bases, such as e.g. lactate, ammonium or CO_2, in the culture medium. Correlations between pH_i and cell cycle phases have also been described. Thus, in general, a more alkaline pH value is measured in the cytosol if the cell is in a metabolically active phase of the cell cycle (S and/or G2 phase, respectively) (Welsh and Al-Rubeai, 1996).

The intracellular pH value can be determined flow cytometrically using a pH-sensitive fluorescence dye that is stimulated in the cell. The fluorescence dye used in the present invention 5'(and 6')-carboxy-10-dimethylamino-3-hydroxyspiro[7H-benzo[c]xanthene-7,1'(3'H)-isobenzofuran]-3' one, often referred to as carboxy seminaphtorhodafluor acetoxymethyl ester or simply carboxy-SNARF-1-AM, in the following referred to as SNARF-1) is a weak acid with a pK_a value of 7.3-7.4 at 37° C. and, upon excitation with light of a wavelength of 488 nm, exhibiting two fluorescence signal maxima of different wavelength (FL2: 580 nm, FL3: 640 nm, FIGS. **3-7**) (C-1270, Molecular Probes). The relative correlation of these emission maxima shows a pH-dependent shift and, thus, allows to measure pH_i independently of the amount of dye excited in the cell.

Being an acetoxymethyl derivative, the fluorescence dye is cell membrane dependent and is hydrolysed in the cytosol of unspecific esterases. This free-form fluorophor remains in the cell and can be exited for emission in the cell. In order to determine the intracellular pH value, a calibration curve must be established by implication of known pH_i values. It must be based on the same cell population from which the sample to be measured is derived (Owen, 1991; Owen, Carango et al. 1992). In this context, the maintenance of the reactor conditions during the preparation of the samples and the selection of the calibration method are of equal importance (Cherlet, Franck et al., 1999; Bond and Varley, 2005; Jockwer, Gätgens et al., 2005) in order to obtain representative pH_i values.

In the present invention, the pseudo zero-point calibration method which is based on the deflection of the stationary pH_i by adding weak acids and bases in known mixture ratios to the cells was used to calibrate the intracellular pH value. According to this, the resulting fluorescence ratio reflects the new pH_i. If the molar concentration of the mixture of a weak acid and base was sufficiently high, a further addition of the same mixture will not alter either the fluorescence ratio or the pH_i, so that the deflected pH_i represents a new pseudo zero-point satisfying the equation 3-3. If concentrated mixtures of different molar ratios of weak acid to weak base are added to the cells loaded with the pH-sensitive fluorescence dye are, it is possible to establish a calibration curve by plotting the pseudo zero-pH against the fluorescence ratios obtained. This cali-

bration method can be reproduced flow-cytometrically and requires only little time for implementation, it is independent of the intracellular K^+ concentration and, thus, suitable for analysis in the course of the fermentation process.

For representative sampling from bioreactors (with pressure blanketing) and cultivation with defined compositions of dissolved gasses, a specific sampling (Jockwer, Gätgens et al., 2005) was developed which is the result of the present invention. The parameters which are relevant for the pH_i during the period of dye absorption such as pCO_2, pressure, pH and temperature are entered into this device so that the cell sample is stained under reproducible reactor conditions. Thus, the pH_i measured in said manner represents the real conditions in the bioreactor better than conventional methods. Together with the stained sample, an unstained sample of the same population was studied as a control under identical conditions.

Solutions and Buffers

HEPES buffer (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]			(10 mM)
2.383 g L^{-1} HEPES			(10 mM)
7.802 g L^{-1} NaCl			(133.5 mM)
0.298 g L^{-1} KCl			(4 mM)
0.166 g L^{-1} $NaH_2PO_4 \bullet H_2O$			(1.2 mM)
0.144 g L^{-1} $MgSO_4$			(1.2 mM)
1.981 g L^{-1} α-D-glucose			(11 mM)
0.294 g L^{-1} $CaCl_2 \bullet 2H_2O$			(2 mM)
pH 7.4, sterile filtration, storage at 4° C.			
dye SNARF-1 (carboxy seminaphtorhodafluor acetoxymethyl ester)			(2 mM)
50 µg	carboxy-SNARF-1-AM (C1272, Molecular Probes, Leiden, The Netherlands)		
44 µl	DMSO		
Butyric acid BA	1M		
(n-butyric acid)	4.6 mL	butyric acid 10.9M	(pK_a = 4.82, Sigma)
	ad 50 mL	H_2O	
	pH 7.4, sterile filtration, storage at 4° C.		
trimethylamine	1M		
TMA	12.3 mL	trimethylamine 4.06M	(pK_a = 9.8, Sigma)
	ad 50 mL	H_2O	
	pH 7.4, sterile filtration, storage at 4° C.		
HDFBS	10% (v/v)	dialysed FBS in HEPES buffer	

TABLE 1

Pseudo-zero calibration solutions (Chow, Hedley et al., 1996)

6-fold concentrate BA/TMA [mM]	0.5 log ([BA/TMA])	BA [µL]	TMA [µL]	HDFBS [mL]
6/96	−0.6	60	960	8.98
6/24	−0.3	60	240	9.70
6/6	0	60	60	9.88
24/6	0.3	240	60	9.70
96/6	0.6	960	60	8.98

Prior to sampling from the relevant culture system by means of the sampling apparatus developed (Jockwer, Gätgens et al., 2005) the 6-fold concentrated pseudo-zero calibration solutions (Table 3-1) were placed into measurement tanks of the flow cytometer (200 µL each), the tanks were closed in a gas-tight manner and stored in a cool place (4° C.). The two calibration solutions of the highest and of the lowest mixture ratio of acid to base were prepared twice each for the adjustment of the measuring ranges of the flow cytometer. For the sample that was to be determined, 3 measuring tanks were treated with HDFBS (200 µL per tank) in the same manner. Prior to collecting the sample from the culture medium, 22 µL of the fluorescence dye were placed into the pre-incubated sampling apparatus (37° C.). The staining of the cells in esterase-free culture medium was started by mixing the cell suspension during the isobaric and gas-tight sampling and was continued in darkness under agitation (appropriate reactor pressure, 25 min, 37° C.). After 15 minutes incubation, the temperature of the calibration and measuring solutions, still sealed and gas-tight, is adjusted to reactor temperature (water bath, 37° C.). Once the incubation of the cells with the fluorescence dye under reactor conditions is terminated, the cells are immediately subjected to pressure control and dissolved gas analysis (Compact 3, Roche Diagnostics GmbH) and subsequently suspended in the corresponding measuring and calibration solutions (200 µL each) to be measured after 10 seconds by means of a flow cytometer (FACSCalibur, Becton Dickinson). For this purpose, first a repeat determination of the sample to be determined is carried out in measuring buffer (HDFBS) and only then the stained cells are measured sequentially in the corresponding measuring buffers. The pH value of each sample is measured (Compact 3, Roche Diagnostics GmbH) immediately after the flow cytometric measurement of this sample.

The time elapsing between the opening of the sampling apparatus and the dissolved gas analysis of stained and unstained cells is less than 5 seconds, the time elapsing until the flow cytometric measurement is terminated is less than 90 seconds. Between the termination of the fluorescence analysis and the determination of the external pH value, 15 seconds elapse. This procedure in combination with the (pressure maintaining) sampling apparatus allows to determine a representative pH_i value (see also Chapter 4.2) (Chow, Hedley et al., 1996).

Measurement

The flow cytometric measurement of the fluorescence to determine the pH_i values is carried out according the manufacturer's instructions for the device (FACSCalibur, Becton Dickinson), the dye (Carboxy-SNARF1-AM, Molecular Probes, Leiden, The Netherlands) and according to the relevant literature (Chow and Hedley, 1997).

The basic settings which must be optimized for each sample are listed below:

measurement window 1: SSC/FSC (scatter diagram, scaling double linear, total cell population)

measurement window 2: counts (linear)/FL2 (log.) (histogram)

measurement window 3: counts (linear)/FL3 (log.) (histogram)

measurement window 4: counts (linear)/fluorescence ratio (FL2/FL3)

Dead cells, agglomerations and insufficiently stained cells were excluded from the measurement by suitable limiting values. Since it is not possible to implement measurement window 4 in real time with the measuring device used, the fluorescence ratio is calculated using the program FCSassist (Becton Dickinson). The program CellQuest 3.3 (Becton Dickinson) was used for the evaluation. Plotting the "mean fluorescence intensity ratio" (MFI) against each induced pH_i value according to equation 3-3 results in the calibration curve which allows to calculate the pH_i of the sample.

(6) Dissolved Gases, pH Value and Depending Parameter

The blood gas analyzer AVL Compact 3 (Roche Diagnostics GmbH) is used for direct evaluation of the dissolved gases CO_2 and O_2, the pH value and the calculation of the dependent concentrations of hydrogen carbonate and "total CO_2" from the cell-containing culture samples (>55 µL, 37° C.) accord-

ing to the manufacturer's instructions (Roche 2003). After placement of the relevant physiological sample, the device automatically determines the above-mentioned parameters. For this purpose, the following miniaturised electrodes are installed in a tempered measuring chamber:

pH reference electrode
pH measuring electrode
pO_2 measuring electrode
pCO_2 measuring electrode

The direct pCO_2 measurement is a modification of pH measurement. Via a membrane that is impermeable to ions, only gaseous CO_2 gets into the measuring buffer the pH of which is modified by dissociated CO_2, is measured and, after amplification, is indicated as pCO_2 value [mmHg]. The conversion into [%] pCO_2 and/or pO_2, respectively, is carried out separately after the automatic measurement of the atmospheric pressure which is carried out for each sample measurement. Calibration gases and buffer solutions supplied to the device allow for automated calibration and cleaning according to defined cycles.

(D) Cultivation of Animal Cells

All operations were carried out in compliance with the standard operation regulations and directives for the handling of genetically modified organisms, security level S1.

(E) Bioreactor Cultivation

All tests were carried out in commercially available stirred reactors with a working volume of 1 L (Applikon Biotek, Knüllwald) and 10 L (B. Braun Biotech International, Melsungen), respectively, under sterile conditions. All measured values were transferred to the process computer via measurement amplifiers and analog-to-digital coder (SMP Interface, Siemens) and processed using the process control software LabView (National Instruments, USA) and stored. The following process parameters were identical in both systems throughout the cultivation period and were constantly monitored.

standard fermentation conditions (1 L & 10 L reactors)
stirring velocity 200 rpm
temperature 37° C.
dissolved oxygen saturation 40% (in relation to air)
pH 7.0
ratio gassing (1 L reactor: 2.4-5.0 Lh^{-1}; 10 L reactor: 30-45 Lh^{-1})

The mixture of high-grade gas fractions of nitrogen (N_2), oxygen (O_2) and carbon dioxide (CO_2) for the maintenance of the controlled concentration of dissolved oxygen pO_2 with constant gassing rate is referred to as "ratio mode".

(1) Measurement of the Process Parameters

Dissolved Oxygen (pO_2)

The in situ measurement of the concentration of dissolved oxygen in the different aqueous solutions was carried out by means of oxygen electrodes according to Clark (InPro 6000, Mettler Toledo, Urdorf, CH).

Dissolved Carbon Dioxide (pCO_2)

The in situ measurement of the concentration of dissolved carbon dioxide in the different aqueous solutions and during fermentation was carried out by means of the commercially available probe YSI 8500, Yellow Springs Instruments (Yellow Springs, USA) and/or, for comparison measurements in the cell-free system, by means of the probe InPro 5000, Mettler Toledo (Urdorf, CH). Both devices were operated in display mode [% CO_2]. In this context, the indication [%] refers to the equilibrium conditions between CO_2 rate [%] in the gassing mixture and aqueous culture medium under standard fermentation conditions (37° C.) which were set under calibration conditions.

The calibration conditions used and the display values of CO_2 [%] allow the calculation of the equilibrium concentrations of dissolved CO_2 during the process. As in other publications in this technical field, the term pCO_2 [%] is used herein as a synonym for "dissolved concentration" and "partial pressure" of CO_2 though the absolute concentrations of CO_2 actually dissolved in the culture medium have to be calculated on its basis (Zhangi, Schmelzer et al., 1999; Pattison, Swamy et al., 2000; Schmelzer, de Zengotita et al., 2000). 1% saturation corresponds to approximately 7.5 mm Hg, 10% correspond to approximately 75 mm Hg.

Exhaust Gas Analysis

Oxygen and carbon dioxide quantification in the nonaqueous exhaust air of both processes is carried out using the exhaust analyzer Xentra 4900 (Servomex, Hamm). The oxygen rate is measured paramagnetically (0-100% v/v), the carbon dioxide rate is measured by infrared spectroscopy (0-25% v/v).

The pH value is measured using a single-rod pH measurement assembly with gel electrolyte (Applisens, The Netherlands). In contrast to conventional pH electrodes with liquid electrolyte, the pH electrode used exhibits, after autoclaving, an internal pressure which considerable reduces the permeation of the glass membrane by extraneous substances and the electrode drift associated therewith. Prior to installation and sterilisation, the pH electrode is calibrated to two defined pH values (pH 4.00 and pH 6.96) at 37° C. according to the manufacturer's instruction. Prior to starting the fermentation, correct operability was ensured by external comparison measurements with the blood gas analyzer (Chapter 3.3.4). Process overpressure is measured by means of two pre-pressurized gel-filled electrodes installed in the cover of the culture vessel (B. Braun International, Melsungen; Bioengineering AG, Wald, CH). The temperature in the culture vessel is measured at all operation stages by means of a PT 100 resistance thermometer of the reactor manufacturer installed in the probe ring.

(F) Mathematical Methods

For cultivation and fermentation, parameters allowing the description of growth and metabolic rates are determined by analytic methods. Cell densities are indicated in animal cell culture in contrast to microbial systems. In general, dry biomass is not of interest.

Viability describes the rate of viable cell density ZDL with respect to total cell density ZDG:

$$Viabilität = VIA = \frac{ZDL}{ZDG} \cdot 100\% \qquad \text{(equation 3-6)}$$

All cell density dependant values determined in the present invention refer to the viable cell number ZDL. It can be assumed that the contribution of non-viable cells to metabolism is not substantial. Viability is a measuring value with respect to the state of a culture. The growth rate WTR indicates the number of divisions taking place in one time interval.

$$v = WTR = \frac{n}{t_2 - t_1} \qquad \text{(equation 3-7)}$$

WTR=cell division rate, h^{-1}
n=number of division steps during period of time t_2-t_1
t=time, h

The cell number increase is calculated based on the number of division steps as follows:

$$N_2=N_1 \cdot 2^n \quad \text{(equation 3-8)}$$

N=number of cells, total number of cells or number of viable cells

n=number of division steps

Solved for n and substituted into equation 3-8, the equation for the growth rate reads:

$$WTR = \frac{\log N_2 - \log N_1}{\log 2 \cdot (t_2 - t_1)} \quad \text{(equation 3-9)}$$

The specific growth rate μ is calculated by correlating growth velocity with viable cell density.

$$\mu = \frac{1}{ZDL} \cdot \frac{d(ZDL)}{dt} \quad \text{(equation 3-10)}$$

μ=specific growth velocity, h^{-1}

ZDL=cell density, viable, cells mL^{-1}

t=time, h

For the increase in the number of viable cells it is true that:

$$ZDL_2=ZDL_1 \cdot e^{\mu \cdot (t_2 - t_1)} \quad \text{(equation 3-11)}$$

Thus, for μ it is true that:

$$\mu = \frac{\ln ZDL_2 - \ln ZDL_1}{t_2 - t_1} \quad \text{(equation 3-12)}$$

Specific substrate consumption rates can easily be calculated for discontinuous processes. For pulsed feed (bolus) in fed-batch processes, the same correlations can be used since the duration of feeding (<15 s in the experiments of the present invention) and, thus, substrate consumption by the cultured cells during this period of time decreases towards zero. It is however necessary to include any modifications in substrates, metabolites and fermentation volumes resulting from feeding and to adjust them mathematically. (Cell-)specific consumption and formation rates are often the only factors allowing the comparison of different processes.

It is true that:

$$qS = \frac{dS}{dt} \cdot \frac{1}{ZDL} \quad \text{(equation 3-13)}$$

qS=specific substrate consumption rate, mol

S=substrate concentration, mol L^{-1}

t=time, h

ZDL=cell density, viable, cells/mL

The mean cell number in the period of time Δt (t_2-t_1) is determined by the logarithmic mean if the time interval between two sampling points is sufficiently long:

$$\overline{ZDL} = \frac{ZDL_2 - ZDL_1}{\ln ZDL_2 - \ln ZDL_1} \quad \text{(equation 3-14)}$$

$\overline{ZDL}$=mean of cell number, viable, cells mL^{-1}

The integration of equation 3-13 under consideration of equation 3-14 results in:

$$qS = \left(\frac{S_2 - S_1}{t_2 - t_1}\right) \cdot \frac{1}{\overline{ZDL}} \quad \text{(equation 3-15)}$$

The specific product formation rate is calculated analogously to the substrate consumption rate:

$$SPR = qPRO = \left(\frac{PRO_2 - PRO_1}{t_2 - t_1}\right) \cdot \frac{1}{\overline{ZDL}} \quad \text{(equation 3-16)}$$

SPR=qPRO=specific product formation rate, g $cell^{-1} \cdot h^{-1}$

PRO=product concentration, g L^{-1}

Yield Coefficient

The correlation between consumption and formation rates of substrates and products, respectively, is indicated by the yield coefficient Y.

$$Y(A/B) = \frac{qA}{qB} \quad \text{(equation 3-17)}$$

Y(A/B)=yield coefficient for A in correlation to B

qA=cell-specific rate for substance A, mol $cell^{-1} \cdot h^{-1}$

qB=cell-specific rate for substance B, mol $cell^{-1} \cdot h^{-1}$

Chemostat

For a continuous process, the volume flow into the reactor is equal to the volume flow from the reactor. In chemostat processes, the cell size distribution in the volume flow extracted corresponds to the cell size distribution in the reactor. Culture volume is constant. When calculating the rates, flow rate D has to be taken into consideration.

$$D = \frac{F}{V_R} \quad \text{(equation 3-18)}$$

D=flow rate, h^{-1}

F=flow, L

V_R=culture volume, L

Mean retention time of a volume element in the reactor is defined as retention time τ.

$$\tau = \frac{V_R}{F} \quad \text{(equation 3-19)}$$

τ=retention time, h

Thus, flow rate is the reciprocal of retention time.

Consequently, for chemostat, it applies that, in equilibrium, growth rate is adjustable via flow rate, it is true that: μ=D.

With respect to substrate consumption in continuous systems with constant culture volumes, it is true that:

$$\frac{dS}{dt} = D \cdot (S_i - S_0) - Q_S \quad \text{(equation 3-20)}$$

S_i=substrate concentration in the culture, mol L^{-1}
S_0=substrate concentration in the feed, mol L^{-1}
Q_s=volumetric consumption rate, mol L^{-1}
and consequently:

$$Q_S = D \cdot (S_i - S_0) - \left(\frac{S_1 - S_2}{t_2 - t_1}\right) \quad \text{(equation 3-21)}$$

In order to calculate the specific substrate consumption rate, the number of cells in form of the logarithmic mean is additionally included in the calculation.

$$qS = \frac{1}{ZDL} \cdot \left[D \cdot (S_i - S_0) + \left(\frac{S_2 - S_1}{t_2 - t_1}\right)\right] \quad \text{(equation 3-22)}$$

qS=specific substrate consumption rate, mol $cell^{-1} \cdot h^{-1}$
$S_{1,2}$=substrate consumption for $t_{1,2}$, mol L^{-1}

Specific productivity is calculated analogously to the substrate consumption rate:

$$qPRO = \frac{1}{ZDL} \cdot \left[D \cdot PRO + \left(\frac{PRO_2 - PRO_1}{t_2 - t_1}\right)\right] \quad \text{(equation 3-23)}$$

qPRO=specific product forming rate, g $cell^{-1} \cdot h^{-1}$
$PRO_{1,2}$=product concentration for $t_{1,2}$, g L^{-1}

The instantaneous space-time yield in continuous processes is calculated as follows:

$$RZA = \frac{PRO \cdot D}{V_R} \cdot 24 \quad \text{(equation 3-24)}$$

RZA=space-time yield, $gL^{-1} \cdot d^{-1}$

Oxygen Uptake Rate OUR

Due to the relatively low growth rates of animal cell cultures, the high volume flows used in the present invention for gassing the culture and the high control performance of the developed PID controller for dissolved oxygen, it is possible to assume equilibrium conditions for oxygen balancing (Frahm, Blank et al., 2002). Thus, the oxygen uptake OUR can be equalised to the oxygen transfer rate OTR for transfer from the gas phase into the liquid phase and results from equation 3-25,

$$OUR = OTR = k_L^{O_2} a \cdot (c_{in}^{O_2} - c_1^{O_2}) \quad \text{(equation 3-25)}$$

$k_L{}^{O_2}a$=volume-related oxygen transfer coefficient, h^{-1}
$c_{in}{}^{O_2}$=oxygen concentration in the gassing mixture, mol$\cdot L^{-1}$
$c_1{}^{O_2}$=controlled concentration of dissolved oxygen, mol$\cdot L^{-1}$

The concentration of dissolved oxygen and dissolved carbon dioxide and the oxygen and carbon dioxide concentrations of the gassing mixture are control and correcting variables, respectively, and are registered by the process control system. The volume-related oxygen transfer coefficient was determined earlier for the relevant process conditions by experiment.

Carbon Dioxide Evolution Rate CER

Due to the controlled pCO_2 values and simultaneous pH control in the culture medium, CO_2 is not accumulated in the culture medium. Thus, the balance can be established analogously to dissolved oxygen balance (Frahm, Blank et al., 2002).

$$CER=CTR=0.89 \cdot k_L a^{O_2} \cdot (c^{*CO_2} - c_1^{CO_2}) \quad \text{(equation 3-26)}$$

with
$k_L a^{CO_2}$=0.89$\cdot k_L a^{O_2}$=volume-related carbon dioxide transfer coefficient, h^{-1}
c^{*CO_2}=saturation concentration of carbon dioxide in the medium, mol L^{-1} (calculated on the basis of the carbon dioxide concentration of exhaust air)
$c_1{}^{CO_2}$=controlled dissolved carbon dioxide concentration, mol$\cdot L^{-1}$

Respiratory Quotient RQ

The correlation of cell-specific carbon dioxide evolution rate qCER with cell-specific oxygen uptake rate qOUR is referred to as respiratory quotient RQ.

$$RQ = \frac{qCER}{qOUR} \quad \text{(equation 3-27)}$$

RQ=respiratory quotient
qCER=cell-specific carbon dioxide evolution rate, mol
qOUR=cell-specific oxygen uptake rate, mol $cell^{-1} \cdot h^{-1}$

Example 2

Optimization of pCO_2-Associated Process Parameter

In industrial cell fermentation facilities there are hydrostatic pressures up to 350 mbar in the bottom region (filling point/level 3.5 m) due to the different reactor heights of stirred reactor. Due to reasons of sterility, the fermenters are additionally subjected to pressure blanketing involving overpressures of up to 300 mbar so that in production fermenters of 10 m^3 working volume total overpressures of up to 650 mbar could occur in the bottom region. With this overpressure, the CO_2 which is produced by the cells accumulates in the medium and can reach inhibiting levels with high density fermentations if the dissolved carbon dioxide discharge has not been optimized via gassing.

For the simulation of the production scale and for the uncoupled analysis of the involved parameters pressure and content of the dissolved carbon dioxide—synonymously referred to as pCO_2—with bioprocesses involving animal cells, a controller system has been established in analogy to the concentration of dissolved oxygen pO_2. In this context, the control of the content of the dissolved carbon dioxide in the medium should be possible under different pressure conditions, the correcting variable of the pCO_2 control system being transferable to bioreactors. Thus, gassing rates, stirring velocities and culture medium were based on industrial parameters.

(A) Control of the Content of the Dissolved Carbon Dioxide in the Culture Medium

(1) Set-Up and Procedure of the pCO_2 Control

According to standard means, the reaction systems used were supplied with submersible ratio gassing. For the pCO_2 control, their CO_2 mass flow controllers (MFC, Type Brooks 5850 E CO_2, 0-5 $L \cdot h^{-1}$) were actuated by implemented PID controllers in the process control system LabView (Texas Instruments). In addition, only with the 10 L stirred reactor an N_2 mass flow controller (Brooks 5850 TR N_2 0-15 $L \cdot h^{-1}$) was installed in the gassing section so that the gassing volume flow rate for pCO_2 saturation can be increased from 30 $L \cdot h^{-1}$

to a maximum of 45 $L \cdot h^{-1}$. The measurement of the control variable pCO_2 in the medium was carried out by means of an in situ pCO_2 probe YSI 8500 (Yellow Springs Instruments) every 15 seconds discontinuously (chapter 3.5.3.2).

The PID controllers implemented for the control variable in the process control system (LabView, National Instruments, USA) pCO_2 work in a cascaded manner (FIG. **1**). In the case of a positive deviation from the pCO_2 set value, the CO_2 content in the ratio gassing is reduced correspondingly by the controller PID 1 (FIG. **1**). If, despite the completely closed CO_2 MFC, the controller PID 1 does not successfully control the pCO_2 set value in the case of a positive deviation (e.g. by produced CO_2 of the cells), the cascaded controller PID 2 actuates an additional N_2 mass flow controller and appropriately increases the total volume flow rate of the gassing by 15 $L \cdot h^{-1}$ at the most (FIG. **1**).

Thus, the pCO_2 controller described herein is suitable for control of a set value for the concentration of dissolved carbon dioxide. By means of this pCO_2 controller it is now possible to adjust defined pCO_2 values during the whole culture process and to analyse their effect on the cultivated cells. According to Henry's Law, the solubility of a gas in liquid is proportional to its partial pressure in the gas phase above. Due to the controller, accumulation of CO_2 in the medium is possible by increasing the CO_2 ratio in the supply air. With the cultivation period progressing and the cell density in the reactor increasing, the ratio of CO_2 produced by the cells increases in the medium. Consequently, the controller reacts by reducing the CO_2 ratio in the supply air by the controller. If the volumetric carbon dioxide evolution rate (CER) of the cultivated cells is so high that by a complete reduction of the CO_2 ratio in the supply air down to naught the control variable of pCO_2 can no longer be reached, the cascaded controller increases the total volume flow of the gassing and, thus, the transfer of CO_2 from the liquid phase into the gas phase.

(2) Control Performance of pCO_2 and pO_2 Control

The increased gas volume flow by the addition of nitrogen in the supply air for an improved discharge of CO_2 takes effect as a disturbance variable on oxygen control. By means of the control of the set values shown in the following, the suitability of the pCO_2 control together with the pO_2 control was analysed both in the 1 L and in the volume. The interaction of the controllers with the pH controller using different titration agents is described below.

The initial parameters of the controller settings were determined according to the recommendations by Ziegler and Nichols (Rake, 1993). For this purpose, the PID controllers of both control systems were configured as simple P controllers (hold-back time T_V=0, reset time T_N=∞). Variation of the proportional region X_P up to the critical system enhancement of the respective control system results in $X_{P,\ crit}$. The period of controlling oscillation with $X_{P,\ crit}$ corresponds to the time T_{crit}.

TABLE 2

Recommended controller parameters according to Ziegler and Nichols at stability margin

PID ratio	setting value
P	$X_P \approx 1.7\ X_{P,\ crit}$
I	$T_N \approx 0.5\ T_{crit}$
D	$T_V \approx 0.12\ T_{crit}$

By means of an appropriate control of the set values (Table 2) of both control systems, the quality of control for the use in fermentations of animal cells was optimized. Both control systems work independently from one another with minimal initial oscillating time and control deviation. It was possible to minimise the effect of the increased volume flow on the control of the dissolved oxygen with increased CO_2 discharge (FIG. **4-2** and FIG. **4-3**).

TABLE 3

Set value profile for the qualification of control in FIG. 2 and FIG. 3

set value		time
pCO_2 [%]	pO_2 [%]	[h]
5	40	|
15	40	|
15	50	|
10	30	|
5	40	↓

The absolute control deviation for dissolved oxygen in the case of set value variances (which do not occur in that manner under cultivation conditions) in processes with low overpressure (50 mbar) is less than 2%: Analogously, the control deviation for the dissolved carbon dioxide ratio is <1% (absolute) with set value variances of absolute 5-10%, which are unusual in fermentation processes (FIG. **2**). Also in the case of a reactor involving pressure blanketing (750 mbar) for the simulation of high filling levels in industrial production facilities and the thus increased solubilities of CO_2 and O_2 in the liquid phase, the quality of the implemented control is sufficient for animal cell culture processes (FIG. **3**).

With the set value control performed according to Table 3, the dissolved oxygen value alone temporarily exceeded the set value (<4% absolute) if the total volume flow is increased for discharge of the dissolved CO_2 (FIG. **3**). The control of pCO_2 with deviations of <1% absolute was very exact. Due to a time-pending control of the set value of the pCO_2, it was possible to reproduce the defined profiles from production fermenters onto a small scale.

In the following the pCO_2 and pO_2 controllers described herein were optimized for the decoupled control in combination with the implemented overpressure controller in order to be able to reproduce large-scale fermenters with regard to these process parameters.

(B) Overpressure Controller

There is a trend towards large-scale production facilities also in animal cell fermentation so that for the simulation of the different hydrostatic pressure conditions from industrial production scales a pressure control system up to 1000 mbar overpressure was developed for the stirred reactor with a steal vessel used in the present invention (10 L working volume, B. Braun, chapter 3.5.2). This reactor was meant to control defined set values independently from the controllers for pCO_2, pO_2 and pH value.

(1) Set-Up and Functionality

At the non-sterile side of the exhaust air line of the fermenter a pressure control valve (Brooks pressure controller, Model 5866 Series, 0-68 $L \cdot h^{-1}$) was installed. The opening width was the actuating variable for the developed PID pressure controller in the process control system (LabView). The control variable was measured on the inside of the reactor lid by means of a gel filled pressure sensor and transferred to the process control system. Due to the rapid decrease in pressure even with small opening widths of the pressure control valve in contrast to the slow increase in pressure due to the low gassing rate with closed pressure control valve, it was not possible to control the control process within the required

tolerance. Thus, in addition, a membrane valve was installed further down the exhaust air line in the direction of the exhaust analyser, whose valve position could be preset manually (FIG. **4**).

The set-up developed in said manner, i.e. with an additional membrane valve, has the following advantages:

- safety margin: Maintenance of an adjustable minimum pressure in case of failure of the pressure control valve during operation
- sufficient inertia of the control process despite sub-optimum flow rate of the pressure control valve (maximum possible valve throughput 68 $L \cdot h^{-1}$, maximum gassing rate 45 $L \cdot h^{-1}$)

Due to the variable gassing rates necessary for pCO_2 control, installation and manual adjustment of the pressure by means of the membrane valve alone was not possible.

(2) Quality Control of Overpressure Control

Control optimization was carried out according to Ziegler and Nichols (Rake, 1993). The operation capability of the overpressure control of a static set value with simultaneous control of pCO_2 set value profiles was furthermore complemented by an automated, process time dependent control of the set value. This allowed for the generation of defined overpressure profiles for, e.g., the simulation of mixture profiles prevailing in large-volume production fermenters. Due to mixing times of partly several minutes, the mixing profiles in these reactors deviate from the model of homogenously mixed reactors and according thereto different spatial positions of the suspended cells in the hydrostatic pressure profile. FIG. **5** shows an example of a time-dependent pressure profile in the 10 L stirring vessel based on a pressure profile of a production fermenter. At the same time a dynamic control of the pCO_2 set value is shown which can be carried out independently from the pressure profile adjusted.

The control of the intermittent set value of the reactor overpressure p (each for 30 min at 750 mbar and 1 h at 450 mbar) between the process times t=20 h and t=26 h shows a high quality of control (±30 mbar) without having significant effects on the control of the set value of the dissolved carbon dioxide concentration (<1% absolute) (FIG. **5**). This is particularly important when considering the increasing solubility of the CO_2 in the case of increase in pressure. Likewise, changes of the pCO_2 set value do not show any changes of the quality of control of overpressure in the course of the process (FIG. **5**) despite the fact that e.g. for decreasing the pCO_2 ratio in the fermenter medium by increased nitrogen flow, the total gas volume flow into the fermenter was increased by 50%. Set values between 1.5-25% pCO_2 with up to 1000 mbar overpressure could be adjusted and corrected in this set-up.

It was shown that it is possible to control oxygen, pressure and pCO_2 independently from one another and with high quality of control. Despite constant, low rotational speed of the stirrer the oxygen entry for animal cell cultures is sufficient. Due to appropriate control of the set value, the simulation of the enrichment of CO_2 is possible during the course of the process. Also, a profile for the simulation of changes in pressure due to hydrostatic effects in high production reactors can be adjusted. Thus, in total, it is possible that the existing requirements for the industrial process parameters considered can be implemented dynamically.

(C) pH Adjustment Agents

In the excess metabolism cultivated cell lines produce significant amounts of lactate and other organic acids which, together with the CO_2 discharged into the medium, lower the pH value of the culture medium in the course of the process. The effect of the pH value in the medium on the cell physiology and product quality (e.g. of secreted glycoproteins) has been examined sufficiently. Thus, in optimized processes, the pH value of the medium is adjusted in defined ranges, mostly between pH 6.6 and pH 7.4, by addition of different bases to the cultivated cell suspension. Common bases are NaOH, which is available for CIP (clean in place) processes in most validated production facilities, and Na_2CO_3 and/or $NaHCO_3$, which is a buffer component in most cell culture media. Often, CO_2 is mixed to the gassing air of cell culture processes in the starting phase to lower the pH value of the medium. In that way, even with media free of $NaHCO_3$ a balance is formed between the CO_2 added during gassing and the aqueous culture medium.

Negative effects of pH correction can occur in large-scale production facilities in the case of addition of strong bases via the head region. In this context, due to long mixing times of up to several minutes, pH gradients of up to 0.8 pH units between instillation site and pH measuring point were described (Langheinrich and Nienow, 1999). The settings of the pH controller also have great effects on cell density and induction of apoptosis in the culture vessel (Osman, Birch et al., 2001; Osman, Birch et al., 2002).

(1) Effect of the pH Adjustment Agent to a Cell-Free System

The basic pH adjustment agents NaOH and Na_2CO_3 were compared with different, simulated cell culture conditions but identical control settings (FIGS. **6** and **7**). Their effect on cultivation of a CHO cell line in fed-batch processes is described in the following.

With identical pH control settings in Biostat ES, controlled 5% pCO_2 were adjusted in an aqueous phosphate/hydrogen carbonate buffer mixture in Biostat (30 $L \cdot h^{-1}$, 37° C., 200 rpm, 750 mbar overpressure). The result was a pH initial value of 7.28. Then, due to a change in the set value in the process control system, a switch of pCO_2 from 5% pCO_2 to 15% pCO_2 was provoked and pH control was started simultaneously. For this purpose, the pH set value was adjusted to 7.2 with NaOH (1 M, FIG. **6**) and/or Na_2CO_3 (1 M, FIG. **7**). These test conditions simulated an accelerated enrichment of the CO_2 produced by the cells in the medium and the pH control with the corresponding base, which is directed against the acidification caused (FIGS. **6** and **7**).

For pH control using NaOH, 2.6 h and 26 dosages were necessary for the change of the pCO_2 set value (FIG. **6**). The resulting dosage frequency is 10 h^{-1}. For the pH control using Na_2CO_3, the pCO_2 setting time was reduced by 60% to 1.6 h (FIG. **7**). The CO_2 additionally entered by the Na_2CO_3 causes reconcentration of the dissolved CO_2 parallel to the pCO_2 controller. In comparison to the NaOH, the dosage frequency (8 h^{-1}) is reduced. The tendency of reduced base consumption with use of Na_2CO_3 instead of NaOH is illustrated by means of the cumulative dosage profile in FIG. **8**. For the pCO_2 set value change described, the double amount of 1 M NaOH is required for pH control. In total, 220 g 1 M NaOH (0.21 mol) in contrast to 110 g 1 M Na_2CO_3 (0.1 mol) were entered into the reactor (FIG. **8**).

Thus, the entry of sodium ions is identical with both titration agents. Thus, a negative effect on the cell culture by potentially different osmotic effects due to use of the bases described can be excluded with pCO_2 control. Since the final conditions of both titration systems are identical due to the equilibrium conditions, the bases NaOH and Na_2CO_3 can have a different effect on the cultivated cells due to the following effects: number of dosages, frequency of dosages, disturbances of the CO_2 balance—extracellular & intracellular.

Osman et al. examined the effect of pH disturbances on the mouse myeloma cell line GS-NS0 in set cultures (Osman, Birch et al., 2002). For this purpose, they exposed the cells to

pH increases to pH 8.0, several times sequentially, as they also occur by suboptimum mixing in production fermenters with addition of strong bases such as NaOH and/or Na_2CO_3 (Langheinrich and Nienow 1999). They found that with increasing number of pH deflections to pH 8.0 the cell viability decreases. Likewise, an increasing frequency of the bases addition to pH 9.0 decreased the cell viability significantly (Osman, Birch et al., 2002). Since with use of NaOH as pH adjustment agent in the present invention, both effects described by Osman et al. play a role (increase in dosage frequency and number of dosages), similarly negative effects on cultivated cells are to be expected. In contrast to the results achieved under the pCO_2-controlled conditions mentioned above, it has to be assumed that the negative effect of NaOH is significantly stronger on cell physiology if pCO_2 control is not used.

It is to be assumed that the strong disturbance of the CO_2 balance in the culture medium by titration with NaOH (prolonged pCO_2 adjustment time, compare FIGS. **6** and **7**) can also have an effect on the cytosole of the cells present in the culture medium. This hypothesis is supported by the findings presented with the development of the pressure-controlled sampling apparatus and those shown in the following as to the effect of pH titration agents on cultivated animal cells on a small scale.

(2) Effect pH Adjustment Agents on Fed-Batch Cultivations of a CHO-K1 Cell Line at the 1 L Scale

The above observations of effects of NaOH and Na_2CO_3 should be evaluated in fed-batch cultivations of the cell line CHO-MUC1 in small-scale fermenter systems (1 L Applikon). The small fermenter described was operated in the Fed Batch Modus with membrane gassing and overpressure to simulate the hydrostatic pressure effects of bigger reactors. The pressurizer unit (provided by Bioengineering) worked autonomously and the pressure was adjusted manually by means of a retention valve behind the exhaust filter. The pressure measurement took place on the sterile side with an adapter piece especially designed by means of a piezosensitive pressure sensor. During pre-fermentation of the cells in the reactor, an overpressure of 60 mbar was maintained to simulate small fermenters of industrial propagation. By means of partial harvesting of the fermenter volume, increasing the pressure to 150 mbar and use of a main fermentation medium, the main production process (100 L) was simulated. By means of feeding medium the glucose ratio was maintained above 1.0 g L^{-1} and the glutamine ratio between 0.8 g L^{-1} and 1.0 g L^{-1}.

In the following, the live cell densities and viabilities achieved in the course of the process are illustrated in the FIGS. **9** and **10** for different CO_2 profiles of the gassing mixture. The results illustrated in FIG. **9** were realised with a constant ratio of 5% (v/v) CO_2 in the gassing mixture. For manual pH control at pH 7.2, however, the CO_2 ratio in the gassing mixture was successively reduced for the fermentation illustrated in FIG. **10**.

For both CO_2 gassing modes, higher viable cell densities, prolonged cultivation periods and higher product concentrations can be achieved when using Na_2CO_3 instead of NaOH (FIGS. **9** and **10**). The lactate concentrations show a continuous increase over the whole course of process for both pH adjustment agents used (FIG. **11**).

Therefore, the resulting cell-specific lactate formation rates are higher when NaOH is used as base than when Na_2CO_3 is used (calculated data not shown). When NaOH is used as adjustment agent, osmolalitites over the threshold of 400 mOsmol kg^{-1} correlate with a slowing down of cell growth, when Na_2CO_3 is used, however, the cells continue to grow exponentially to enter the stationary growth phase at a later point in time (FIG. **12**). High-osmolar processes, as described in the literature as to increase in productivity (Kim, Kim et al., 2002), can be further optimized by use of Na_2CO_3 instead of NaOH as pH adjustment agent, according to the results shown herein. Particularly in combination with the pCO_2 controller developed, processes of that kind can probably be further improved with regard to cell metabolism, cell physiology and product formation. On the basis of these results, Na_2CO_3 was used exclusively as pH adjustment agent for further cultivations in the present invention.

Example 3

Pressure-Controlled Sampling for Fermentation Accompanying Measurement of Cell-Physiological Parameters in Pressure Blanketing Fermentations of Animal Cells

As already described above, industrial fermentations of animal cells are carried out on a large scale where, according to Henry's Law, hydrostatic pressure, in combination with sterilisation-related overpressure, causes increased solubility of gases contained in the culture medium. In particular, the solubility of carbon dioxide produced by the cells and released into the aqueous medium is approximately 25 times higher than that of oxygen under similar conditions (Bailey and Ollis, 1986). The related and easy-to-measure change of the pH value in the culture medium can be minimized by using suitable buffer systems and pH adjustment agents. Since CO_2 is a small unpolar molecule, it is capable to permeate the membrane of animal cells and, thus, influence the intracellular pH value (Thomas, 1995).

In general, cell-containing samples are collected in defined intervals from the culture for fermentation accompanying analysis. According to the state of the art, conventional sampling methods from fermentations with pressure blanketing, envisage opening a valve installed in the bottom region and releasing, due to the overpressure in the culture vessel, a defined volume of cell-containing sample without pressure into a suitable vessel. The related expansion of the cell suspension involves a time-dependent degasification of the suspension. The actual concentrations of dissolved gas in the corresponding fermenters may, consequently, be represented only insufficiently by the blood gas analyzers according to conventional sampling (see below).

The following Examples give results of studies on this matter which, in addition to the extracellular changes in the medium, feature on the effect of the sampling method on the intracellular pH value of the cells contained in the medium. The development of an apparatus for pressure-controlled sampling and the necessity to apply it with analysis of the intracellular pH value of animal cells, which accompanies cultivation in large-scale fermentation and/or fermentation with pressure blanketing, are presented.

(A) Construction and Development of a Pressure-Controlled Sampling Apparatus

(1) Qualification by In Situ and Off-Line pCO_2 Measurements

In order to examine the effect of different sampling methods on the concentrations of dissolved gas in the medium—according to the state of the art and using the apparatus developed—different concentrations of dissolved carbon dioxide and different pressures were set in the 10 L reactor Biostat ES to simulate the different conditions in industrial large-scale fermenters. For this purpose, the appropriate medium was gassed with defined carbon dioxide ratios in the

gassing mixture under standard fermentation conditions (ratio gassing 30 L·h^{-1}, 37° C., 200 rpm) and otherwise the parameters indicated in Example 2(E). In this context, the pH value was not adjusted by the addition of a base. For the measurements, the CO_2 concentration increased by the increasing total pressure in the medium was in equilibrium with the gaseous phase (exhaust analysis. Xentra 4900, Servomex). FIG. **13** shows the parameters the combination of which led to the 30 experiments which were carried out.

The measurements of the dissolved carbon dioxide ratio were carried out using in situ pCO_2 probes (Yellow Springs Instruments, USA) and/or, subsequent to sampling, off-line using a blood gas analyzer (AVL Compact 3, Roche Diagnostics GmbH). FIG. **14** indicates exemplary results for 0.15 M NaCl as aqueous medium. From this, the relatively high loss of dissolved CO_2 between in situ measurement values and conventional sampling methods becomes clear (ΔpCO_2=30%).

The smaller loss of CO_2 with use of the sealed syringe is to be attributed, primarily, to the lower degree of mixing with CO_2-free ambient air which inevitably occurs with conventional sampling. Sampling with the syringe is carried out by means of a sterile adapter which could be connected directly to the sampling valve of the reactor (FIG. **16**). The internal pressure of the reactor moved the plunger installed in a gas-tight manner in the syringe fixed in a support against atmospheric pressure to the preset stop (FIGS. **17** and **18**). Comparative results of this preferred sampling method with different reactor overpressures are shown in FIG. **15**. Further reduction of CO_2 loss was pursued by modification of this sampling method.

The use of this syringe-based reactor sampling technique already allows to minimize the loss of dissolved gas concentration (ΔpCO_2) from 30% to 20%.

The maintenance of the overpressure prevailing in the reactor at sampling time should further minimize the loss of dissolved gas. For continuous measurement of the sample pressure, a piezoelectric pressure receiver was integrated into the sampling line and connected to a digital display (FIG. **16**).

Thus, pressure measurement was also possible after uncoupling the sampling unit from the reactor. Control of the sample pressure indicated on reactor level and, consequently, the isobaric collection of the sample was carried out manually by slowly releasing the (cell-)suspension from the pressure-blanketed fermenter into the sampling unit connected thereto (FIG. **16**). The knurled screws installed allowed fine-tuning of the sample pressure subsequent to uncoupling the unit from the reactor (FIG. **16-18**). The pressure-controlled sampling apparatus (DNP) developed in this way maintained the dissolved gas concentrations in the cell-free sample on reactor level over a period of several hours (Klinger, 2006). FIGS. **17** and **18** show a photography of the support unit for different sample volumes which were obtained using syringes and adapter sleeves and a top-view of the support unit with installed syringe.

At this stage of the development, due to the sterile construction, it was possible to use and qualify the specifically developed DPN for sampling in pressure-blanketed fermentations of animal cells. The sterile couplings (Stäubli, Switzerland) in combination with the steam-sterilisable sampling valve of the bioreactor, the sterile mode of operation of the stirring vessel was not limited (qualifications not shown).

FIG. **19** shows in an exemplary manner, the CO_2 concentration measured off-line according to conventional sampling method in comparison with the pressure-controlled sampling method developed at termination of a pressure-blanketed fed-batch cultivation of the CHO-MUC1 cell line. Even though

the dissolved carbon dioxide concentrations do not significantly differ in the different vessels immediately after sampling, the concentration of dissolved CO_2 in the conventional sampling methods decreases by 20% during the first 12 minutes already. Thus, if conventional sampling techniques are applied, a representative measurement of dissolved CO_2 in fermentations with pressure blanketing is only possible with immediate measurement by means of a blood gas analyzer.

The use of the sampling apparatus developed, while maintaining reactor pressure and reactor temperature, is an essential advantage. The sampling apparatus maintains the initial reactor concentration of dissolved CO_2 over a storage period of one hour (FIG. **19**). In this context, the concentration of dissolved oxygen was not limiting at any time (Klinger, 2006). Due to fact that the pressure and pCO_2 concentrations of the reactor are maintained outside the reactor, the pressure-controlled sampling apparatus developed allows medium-term sample treatment (<1 h) under bioreactor conditions.

In particular, the period of time analysed in FIG. **19** is suited in an optimum manner for the treatment of cell-containing sample with a fluorescence dye as, for example, it is required for the measurement of the intracellular pH value. The fundamental correlations between the pCO_2 level in the culture medium and the intracellular pH value are shown in Example 4, the results of pCO_2-controlled fermentation obtained using the sampling apparatus as described herein are given in Examples 5 to 7.

In the following, exemplary results of flow cytometric studies on cell physiological qualifications of the different sampling methods are presented.

(2) Qualification by Means of Flow Cytometric Measurements

Maintaining the reactor conditions (temperature, pH value, components of the medium, concentrations of dissolved gas, pressure) was considered only to some extent by the flow cytometric determination of the intracellular pH value using conventional protocols according to the state of the art. Little attention was paid, in particular, to the sampling from pressure-blanketed fermentations and the associated degasification of the medium and their effect on the physiology of the cells contained in the sample. From the following Examples, it becomes clear that, even though the extracellular measurement values pH and CO_2 show no detectable changes in the medium after sampling, the cells suspended in the medium can already differ significantly with respect to the physiological conditions in the bioreactor.

By using the developed sampling apparatus in flow cytometric determinations, it is possible to measure intracellular pH values which represent the physiological state of the examined cells in the bioreactor essentially better than it is possible in the state of the art. The following FIGS. **20** and **21** show results from flow cytometric pH_i measurements which were achieved without (FIG. **20**) and with pressure-controlled sampling (FIG. **21**), respectively. In the partial illustrations 2 and 3, to the right of the respective Figure, the intensities of the corresponding fluorescences (FL2-H AND FL3-H, respectively) are plotted against the cell size. The partial illustrations 1, on the left, show the ratio peak resulting from the ratios of fluorescence intensities FL3/FL2. This ratio determines the pH_i value according to the calibration values. Its value is determined by the number of counts per detection channel.

In order to determine the pH_i value of a CHO cell population in the pressure-blanketed fermenter according to the state of the art, first, a sample was collected in a sterile manner under decompression to atmospheric pressure and then incubated with the fluorescence dye SNARF-1 for 25 minutes as

described above. The subsequent measurement in the flow cytometer showed the fluorescence intensity ratios shown in FIGS. **20** A and B. In FIG. **20** A**1**, a double-peak can be observed which is based on two cell populations with different fluorescence intensities (FIG. **20** A**2** and A**3**). Despite the selection of suitable boundaries for the individual peaks (FIG. **20** B, upper populations), it is not possible to relate the peaks unequivocally to the initial pH_i value of the population in the reactor.

Upon observation of the same sample after a period of 50 minutes (FIG. **2**-**20** C), the fluorescence of the cell population has completely shifted causing due to its homogeneity a single peak of the corresponding fluorescence intensity ratio (FIG. **20** C**1**). This single peak represents the final state of a time-dependent change of the fluorescent cell population for which, temporarily, a double population can be observed as shown in FIG. **20** A.

This assumption was to be confirmed by using the pressure-controlled sampling for the incubation of the reactor sample, which was obtained in an isobaric manner, with the fluorescence dye SNARF-1. If the partial populations with high fluorescence intensity (scatter diagrams in FIG. **20** A**2**, A**3**) and the position of the corresponding ratio peaks (FIG. **20** A**1**) after isobaric incubation in the pressure-controlled sampling apparatus are compared with those from incubation without pressure according to conventional sampling (scatter diagram in FIG. **21** D**2** and D**3** and ratio peak FIG. **20** D**1**), the following differences can be observed. Contrary to incubation without pressure (according to the state of the art), even after 25 minutes of incubation, a homogenous fluorescent cell population (FIG. **21** D**2** and D**3**) and a corresponding single ratio peak (FIG. **21** D**1**) can be observed. If the same sample is incubated for further 25 minutes without overpressure, the whole fluorescent cell population is shifted analogously to the one without pressure (cf. FIG. **21** E**2** and E**3**; FIG. **21** C**2** and C**3**). The final positions of the single peaks are identical (cf. FIG. **21** E**1** and FIG. **20** C**1**) after a period of 50 minutes, but they do not reflect the real pH_i of the cell population in the reactor.

This supports the theory that, during incubation without pressure under expansion, cell populations from the reactor are subjected to a time-dependent pH_i change which can be measured by means of the inhomogeneous shift of the fluorescence populations and the transient ratio double-peak. These intracellular changes can be measured before extracellular parameter such as pH and pCO_2 may cause significant changes of the measurement values and, thus, may falsify the measurement of the intracellular pH value of the cell population in pressure-blanketed fermenters considerably.

Al-Rubeai (Welsh and Al-Rubeai, 1996) attributed the time-dependent sub-peak (FIG. **20** A**1**, left peak) occurring due to (intracellular) degasification to the non-viable cell population. Thus, according to their studies, there is a striking correlation between the ratio of non-viable cells and the cell populations which are represented by an analogous sub-peak. This is not confirmed by the results presented herein. In fact, the cell populations used for the above-mentioned studies exhibited viabilities >95% and were in the exponential growth phase without limitations. Early apoptotic signals which coincide with an acidification of the cytosol can be excluded as cause for the ratio double-peak shown herein (FIG. **20**, A**1** left peak). According to this, with respect to the bioprocess, they would imply that the pressure variations observed with conventional sampling induced severe apoptosis analogously to e.g. the repetitive (fed-)batch processes, which, however, cannot be observed. The ratio double-peak as observed herein is transient and, thus, it is to be attributed only to sampling and/or incubation without pressure and the resulting degasification of the cells in suspension.

In fact, in the present invention, ratio double-peaks were also observed with the use of the pressure-controlled sampling apparatus, but only at the end of (fed-)batch cultivations when measurable decreases in viability can be registered (data not shown). These coincide with late phases of the apoptosis which were measured by parallely applied flow cytometry by means of DNA quantification (sub-G1 peak with cell cycle analysis using DNA fragments).

Thus, the results shown herein support the necessity of isobaric sampling with pressure-blanketed fermentations in order to correctly determine the intracellular pH values of the reactor population.

Example 4

Effects of the Content of the Dissolved Carbon Dioxide on the Intracellular pH Value of Animal Cells

As a small unpolar molecule, physically dissolved CO_2, a product of metabolism of cultivated cells, can permeate the cell membrane relatively unhindered without requiring specific transporters (Thomas, 1995). However, within the cell and in the extracellular aqueous medium, it is in equilibrium with hydrogen carbonate for which active transports systems are provided to the cell (Hu, Seth et al., 2007). Thus, a change in the concentration of one of the species always causes a corresponding change of the other species involved in the equilibrium and, consequently, a change of the pH value. If changes in the concentration of dissolved CO_2 and/or hydrogen carbonate occur in the culture medium of the cells, this has always time-dependent effects on the intracellular pH value (pH_i) of the cells. The maintenance of the physiological medium and, thus, the intracellular processes is carried out by the cell via regulation of the pH_i by using transporters in the cell membrane, in part, under energy-consumption conditions (Madshus, 1988; Reusch, 1995).

In the following, the correlation between the pCO_2 content of the culture medium and the pH_i of the cells cultured therein is studied. The CHO-MUC1 cell line was cultured with controlled pCO_2 values in the reactor system (chemostat, 1 L), a sample was collected under maintenance of the reactor conditions and, for the determination of the pH_i, incubated with the pH-sensitive fluorescence dye according to the experiment in question. The concentrations of the pCO_2 in the sample were determined off-line (AVL Compact 3) and the intracellular pH value was measured by means of flow cytometry.

(A) In Vitro Effects of pCO_2 on pH_i

The pCO_2 saturation to the corresponding pCO_2 levels in the medium were achieved by gassing the stained cell suspension with air (AVL Compact 3) immediately (<5 s) before the flow cytometric pH_i measurement. The effects on the intracellular pH value due to different pCO_2 saturations in the culture medium in vitro are shown in FIG. **22**. The parallel graphs of the curves obtained from different reactor samples are indicative for a direct correlation between pCO_2 concentrations in the medium enclosing the cells and the intracellular pH value of the cells. The higher the CO_2 saturation in the culture medium, the higher was the temporary alkalization of the cytosol of the cells contained in the culture medium.

The observed phenomenon of the short-term intracellular alkalization with depletion of dissolved CO_2 in the culture medium can be attributed to the shift of its equilibrium. If the CO_2 which is physically dissolved in the medium and is in

equilibrium with hydrogen carbonate and protons is removed, this will cause an increase in the pH value since the binding of hydrogen carbonate to hydronium ions will increase to replace the dispersing CO_2 with elimination of water. This equilibrium shift takes place in the cytosol so that with decreasing pCO_2 concentration in the medium, the measured pH_i value increases as shown in FIG. **22**. In the following, this short-time effect is referred to as "chemical effect" of the changes of the content of dissolved CO_2 on the intracellular pH value.

As the following studies show, a viable cell actively reacts to this change in its environment. On a deflection from the physiological equilibrium state, as upon the removal of dissolved CO_2 from the culture medium shown in FIG. **22**, in addition to the short-term chemical effect described, the cells always exhibit a reaction in form of a "physiological effect" (FIG. **23**). A biphasic development of the pH_i could be observed in all experiments (FIGS. **23** and **24**). In this context, the short-time alkalization of the cytosol due to CO_2 depletion was always followed by a phase of acidification of the cytosol, which is contrary to the shift of the CO_2 equilibrium. Only 30 to 40 minutes after in vitro CO_2 depletion in the medium, the intracellular pH value of the population dropped below the initial value (FIGS. **23** and **24**). In this context, no significant changes of the extracellular pH value are measured in the cell culture medium used (FIG. **23**), whereas the intracellular pH value shows the above-described biphasic change, with the extracellular pH value being constant.

This super-compensation of pH_i deflection could be observed with different initial pCO_2 values (FIG. **24**), wherein the pCO_2 gradient between the initial concentration in the culture medium and the final value after CO_2 depletion determines the degree of the intracellular alkalization via the "chemical effect" (FIG. **24**).

The super-compensation with an acidification below the initial value if the intracellular pH, which was observed in the second phase, can be correlated with active transport processes through the cell membrane. As already discussed above, there are several relevant transporters in CHO cells which are responsible for pH_i control. They adjust the intracellular pH value by means of a flow equilibrium prior to a dissolved CO_2 disturbance. This phenomenon will be further discussed in the following Example where the in situ effects of CO_2 enrichment in the culture medium on the cytosolic pH value will be examined.

(B) In Situ Effects of pCO_2 on pH_i

The observed in vitro effects of the time-dependent cell response on CO_2 depletion in the culture medium (FIGS. **23** and **24**) were to be simulated in situ in the controlled small-scale fermentation system (Applikon 1 L). For this purpose, the set value profile of pCO_2 was raised gradually during a continuous chemostat cultivation. In FIG. **25**, the resulting pH_i values are plotted for the viable cell density in relation to process time.

As can be seen from FIG. **25**, the above, in vitro observed, effects of the short-time alkalization and the long-time acidification of the cytosol after a decrease of the pCO_2 level in the culture medium also occur in situ with, in contrast, an escalated enrichment of the culture medium in the small-scale fermenter. In this context, corresponding short-time acidification and long-time alkalization can be observed as reactions of the cell to the escalated increase in dissolved CO_2 concentrations in the medium. It must be noted that the long-time alkalizations of the cells after the pCO_2 surge to a higher value (FIG. **25**) remain at this level (+0.1 pH_i units per surge from 2.5% to 5.0% and from 5.0% to 10.0% pCO_2, respectively, in the medium). Thus, the intracellular pH value seems to be directly correlated with the pCO_2 level of the culture medium, since, apart from this parameter, the cultivation conditions are constant in the chemostat process. Thus, there is the possibility to influence the pH_i of the cells, with the described consequences regarding physiology and metabolism, via the pCO_2 concentration in the culture medium.

To date, only incomplete studies on this subject matter have been described in the literature. These examined continuous processes regarding the influence of pCO_2 on animal cells, however, they were based on methods using cell retention which could not ensure a constant cell population (size distribution, cell cycle phase distribution, metabolic consumption and formation rates). Phenomena which can be generally observed are the alkalization of the cytosol with accelerated cell growth and generally intensified metabolism and/or a more acidic pH_i with resting cells (Engasser, Marc et al., 1996; Welsh and Al-Rubeai, 1996). In combination with the pCO_2 controller developed, the chemostat process as studied herein with a cell output over the complete size distribution and with constant cell density provides, for the first time, the possibility of detailed cell physiological and metabolic studies of the cells in equilibrium state. Thus, it is possible to decouple the effects of the pCO_2 content in the medium on pH_i from the changes which, due to cell growth and cell cycle phase distribution, occur e.g. in batch processing or in processing with cell retention and associated size selection.

In the early 1990s, Wu et al. already began to work on single-cell-based computer simulation of the intracellular pH control in CHO cells (Wu, 1993). The "regular model" for cell growth in suspended batch cultures postulated by Wu describes pH_i values for states of equilibrium and alkaline depletions in CHO cells in a satisfying manner, however, the description requires modifications with respect to the pH_i after transient acidification of the cells. The modifications indicated by the authors in this context describe the property of CHO cells allowing activating the NA^+/H^+-antiporter with acute acidification of the cytosol and reducing the activity of said antiporter to a basal level once a new equilibrium is achieved even if the cytosolic pH is still more acidic than the initial pH. The above-mentioned findings from the pH- and pCO_2-controlled chemostat process are contrary to the modifications of the pH_i regulation model of Wu. The effect described by Wu shows high similarity to the "chemical effect", which would be a possible explanation for short-time pHi changes in such simulations. However, once a new in situ equilibrium state is reached in the controlled system, the "physiological effect" prevails with super-compensation by active transport processes and also by activating the NA^+/H^+-antiporters, as postulated by Wu. This "physiological effect" in dynamic situation models of animal cells should be considered in an appropriate manner.

Example 5

pCO_2-Controlled Fed-Batch Cultivation of Cell Line CHO-MUC1 in 10 L-Scale

The findings obtained in the above Examples on the basis of the effect of the different pH adjustment agents, the reactions of the intracellular pH value to the pCO_2 in vitro and in situ and the procedural implementation of the control strategies for pCO_2 and overpressure will be consolidated in the following. For this purpose, the bioreactor Biostat ES was used with the model cell line CHO-MUC1 under industrial conditions (overpressure, pCO_2 profiles, medium, processing mode). Subsequent to revitalisation in spinner flasks, the inoculum was cultivated in a working volume of up to 1 liter

at 5% CO_2 in the incubator. Subsequent to inoculation, the viable cell density in the 10 L reactor was 1.5×10^5 cells mL^{-1}. All further fermentations in 10 L-scale were carried out under standard fermentation conditions as described in Example 1(E) with constant energy transfer. Foaming was counteracted by manually adding an anti-foaming agent (Dow Medical Grade C). The overpressure in the fermenter was adjusted to 750 mbar, the pCO_2 values to constant 5%, 15%, 25% and/or a profile of 5-15% pCO_2, respectively. The dynamic set value profile of 5-15% pCO_2 was intended to simulate CO_2 enrichment in the culture medium during the fermentation process in industrial production processes. According to the relevant literature, 25% pCO_2 should have a deleterious CO_2 effect on the cultivated cells (Kimura and Miller, 1996; Pattison, Swamy et al., 2000; de Zingotita, 2002).

In all cultivations, the concentrations of the control substrates glucose and glutamine was maintained in the range of between 2-5 L^{-1} and 0.5-1.0 g L^{-1}, respectively, by daily pulsed feed, so that amino acid eliminations were prevented. The abort criterion for the fermentation was a viability below 80% in order not to detect erroneously the incompletely translated fusion protein or fusion protein affected by proteases of the cell in ELISA.

(A) Growth Behaviour

FIG. **26** shows growth behaviour and viabilities for the different pCO_2 control profiles. From this, the following tendency becomes apparent. In general, the cultures in the bioreactor grew more rapidly at lower pCO_2 values if these were adjusted to constant set values. At 5% pCO_2, the maximum density of viable cell achieved is highest (1.3×10^7 mL^{-1}) and in minimum time (150 h). In this case, viability decreases comparatively early so that the duration of cultivation is significantly shortened (<200 h). Cultivation with a pCO_2 profile simulating the course of growth in an industrial fermenter on production scale shows a striking course of growth (see also FIG. **28** showing pH_i). In this case, the pCO_2 value is from 5% (start) to 15% (190 h and more) and the related growth curve is between the curves of controlled constant 5% and 15% pCO_2 (FIG. **26**). At 25% pCO_2, the culture shows slower growth, but exhibits higher viable cell densities than the culture at 15% pCO_2. Thus, no toxic effect is observed at 25% pCO_2 yet, a further increase in pCO_2 was not possible due to technical factors. Viability curves and death rates are similar for all controlled constant pCO_2 values (FIG. **26**).

(B) Cell Cycle Phase Distribution

In the following, the cell cycle curves of the G0G1 phase fractions of cultivations with different pCO_2 set value profiles are studied (FIG. **27**). In this context, the relatively high G0G1 fraction at 15% pCO_2 is to be noted which increases from 150 h onward until cultivation is stopped and, thus, reflects a progressing cell arrestation.

The curve of the G0G1 phase fractions for cultivation with a pCO_2 profile of 5-15% is, up to 150 h, analogous to the curve for cultivation that is pCO_2-controlled at constant 5%. Thus, this curve is similar to the curve for 15% pCO_2. This industrial pCO_2 profile shows, however, a particularly distinct turning point at approximately 170 h with the G0G1 fraction decreasing. This correlates with a pCO_2 value of 13% (FIG. **28**). A further increase in the pCO_2 value to 15% and its fixation at this level does not result in a further increase in the G0G1 phase fraction (FIG. **28**). However, in the process which is adjusted to constant 15% pCO_2 since cultivation start, the G0G1 phase fraction further increases until the end of the process (FIG. **27**).

(1) Consequences for Process Development

It remains to be examined whether, in this case, a higher G0G1 phase fraction could be achieved by means of static control of the set value at 13% (turning point G0G1 phase fraction) with the start of cultivation. Thus, a dynamic pCO_2 set value profile—as applied herein or similar—is useful for cell (line)-specific pCO_2 optimization, e.g. by applying the turning point method as shown herein to the curve of cell cycle distributions or other cell parameters (e.g. pH_i, specific rates). Contrary to the profile of industrial large-scale fermenters which is to be simulated, the pCO_2 set value profile as shown herein is applied externally via the control by means of CO_2 addition via gassing. In industrial production fermenters, CO_2 enriched in the medium is provided by the cells themselves. Therefore, it is possible that a corresponding pH_i profile can differ accordingly. This might also be analysed analogously if the pressure-controlled sampling apparatus developed in the present invention is used.

(C) Intracellular pH Value

Since is has been shown that the pH_i value can be influenced by applying the strategy of pCO_2 control, this should also affect cell cycle phase distribution. FIG. **29** shows the G0G1 fractions and pH_i values for the process with controlled constant 25% pCO_2 as a comparison. An acidification process in the cytosol can be observed which coincides with the turning point in the G0G1 phase fraction curve (200 h). Thus, turning points in the pH_i curve of the studied CHO-K1 cell line coincide with turning points in the curve of the G0G1 cell cycle.

In this context, the cell is exposed to a constant pCO_2 value in its environment throughout the whole cultivation process. The pH_i value increases continuously until it reaches a value of 7.35 and, at the turning point of the G0G1 phase fraction curve, continuously decreases until it reaches pH 7.0. A short-time alkalization prior to the decrease of viability and termination of cultivation can also be observed.

In general, at a pCO_2 of this high level, the G0G1 fraction is similarly high at the beginning of cultivation (FIG. **27**) which is reflected by the slow growth behaviour. Throughout the whole fermentation process, the G0G1 phase fraction is within a small range (50-60% G0G1 fraction).

Since intracellular enzyme activities also show pH dependencies, influencing the intracellular pH by means of pCO_2 and the corresponding control strategy is an important process parameter which is to be optimized. Bresnahan and Dittmer correlated, for example, increased specific antibody productivities with increased intracellular pH and cell cycle arrestation, respectively, in the late G1 phase (Bresnahan, Boldogh et al., 1996; Dittmer and Mocarski, 1997). The controller developed in this invention, for the first time, allows to study the decoupling of the pCO_2 level from the pH value of the culture medium and osmolality.

The correlations between pCO_2 with central cell metabolism will be examined in the following.

(D) Glutamine and Glutamate Metabolism

With increasing pCO_2, the glutamine uptake rate is slightly increased, this becomes particularly manifest at 25% pCO_2 (FIG. **30**). Similar starting curves for specific glutamine uptake rates can be observed for 5% pCO_2 and 5-15% pCO_2 as already observed with the G0G1 phase fraction (FIG. **27**).

Based on the yield coefficients for glutamate versus glutamine illustrated in FIG. **31**, it is possible to assume that, in processes with high values of constant controlled pCO_2, the increased glutamine uptake contributes to the formation of glutamate to a lower degree. Y(GLT/GLN) which indicate the correlation between formed glutamate GLT with consumed glutamine GLN are, on average, lower with the constant pCO_2 control value increasing and, furthermore, show different progression curves. With progressing process, a continuous increase of the yield coefficient at a high level can

be observed at 5% pCO_2, whereas, at 25% pCO_2, only a slight increase can be observed at a similarly low level. At 15% pCO_2, the process shows a more constant yield coefficient with a slight tendency to decrease throughout the process. With an increasing pCO_2 set value profile (5-15%), however, the curve of the corresponding yield coefficient is irregular. The local maximum of the yield coefficient at 170 h coincides with the turning point identified before for the G0G1 phase fraction (compare FIG. **31** and FIG. **28**).

(E) Lactate Metabolism

The cell-specific lactate formation rates show the characteristics as depicted in FIG. **32** resulting in cumulative lactate concentration curves as depicted in FIG. **33**. In this context, the cell-specific curve at 25% pCO_2 has to be noticed which shows an increase in the exponential growth phase (70 h-120 h) and again at the end of cultivation (>200 h). The other fermentations examined, in general, show a decrease with respect to the cell-specific lactate formation rate with progressing cultivation. The fermentation at 15% pCO_2 shows the highest specific lactate formation in the stationary growth phase.

(F) Product Formation

The product formations for the processes which are regulated to controlled constant pCO_2 values of 5% and 25% pCO_2 do not show any differences in their progression except with respect to the final antibody concentration MUC1-IgG, which is caused by the varying process durations. The substantially higher productivity of the process which was regulated to an increasing pCO_2 set value profile (FIG. **34**), is significant.

(G) Consequences for Process Development

Thus, in general, with pCO_2 adjustment to controlled constant pCO_2 levels, it is possible to enhance process robustness under the conditions as shown herein. According to the results as shown herein, the adjusted pCO_2 set value profiles, in particular, have potential for cell line specific optimization. Thus, it was possible to increase productivity, under as constant conditions as possible, (e.g. energy transfer, overpressure, temperature, pH value) by increasing the pCO_2 profile. With regard to different industry-related issues, the pCO_2 control as well as the overpressure control developed were simultaneously and successfully used in the 10 L bioreactor in fed-batch processes. The results obtained by pCO_2 control show high potential for the optimization of industrial cell cultivation processes by controlling the following parameters: intracellular pH value, cell cycle distribution, central metabolism (e.g. glucose, lactate, glutamine, glutamate), apoptosis/duration of cultivation.

Example 6

Chemostat Culture of Cell Line CHO-MUC1-IgG: pCO_2 Set Value Control with Glucose Limitation and "Metabolic Shift"

The differences recognized in the central carbon metabolism caused by the different pCO_2 set value profiles were studied in greater detail in the chemostat process (1 L).

In the 1 L bioreactor described in Example 1(E), the recombinant CHO-K1 cell line CHO-MUC1-IgG was cultivated in the chemostat process. For this purpose, gassing was carried out by means of an L-shaped gassing tube (Applikon) which introduced the gassing mixture with constant volume flow into the medium below the propeller stirrer (2.4 Lh^{-1}). In this context, the cell suspension was removed from the near bottom region by means of an immersion nozzle in order to continuously add fresh medium via the head region so that the reactor volume was maintained constant. The corresponding flow rates D and growth rates p are shown in FIG. **35**, viable cell density was within flow equilibrium at 7.5 (±1)×10^6 mL^{-1}.

In the batch phase (<120 h) the substrate glucose is consumed and the culture is delivered to the metabolic switch in order to initiate lactate remetabolisation. In this phase, glutamine is sufficiently available as an alternative carbon source, there were no amino acid limitations. Subsequent to a gradual increase in flow rate D with an increasing number of viable cells, pH control was started at 230 h (from pH 6.6 to pH 7.0 using 1M Na_2CO_3). Thus, the lactate remetabolisation prevailing up to then was stopped and an increased lactate formation occurred.

In the glucose-limited chemostat process, the cell-specific lactate formation rates decreased during the process. However, the growth rate remained equal to the flow rate (FIG. **35**). If there is a set value surge from controlled 10% pCO_2 to 20% pCO_2 (420 h) in this flow equilibrium, the cell-specific lactate formation rate triples during the following 150 h and the glutamine uptake decreases (FIG. **36**). At this position of the gradients between internal and external pH value, on the basis of the findings of the present invention, it is possible to influence the pH_i and, thus, directly influence lactate transport and metabolism via an optimized pCO_2 control (also in combination with pH set value control in the culture medium).

Example 7

pCO_2 Controlled Fed-Batch Cultivations of Cell Line CHO-hGM-CSF-PYC2 on a 1 L-Scale

As proven in the present invention so far, apart from the pH value of the culture medium, also pCO_2 and pCO_2 set value control affect the central metabolism significantly, even under glucose-limited conditions. Furthermore, exact knowledge of the intracellular pH value is essential for a metabolically and energetically optimized cell culture process. Since in particular the metabolic switch for lactate remetabolisation may enable efficient metabolic pathways with an associated increase in productivity in recombinant cell lines, high process robustness desirable for industrial processes. Therefore, in the experiments described herein, the combination of the pCO_2 process engineering developed herein should be carried out using a CHO cell line which is optimized via metabolic engineering and pCO_2-sensitive at the same time. Consequently, a sensitive alignment of the control strategies in the fermenter with the desired cell metabolism is required. In this context, the energy transfer must also be taken into account. In this study, the parameter of the empty tube velocity of gas was selected for pCO_2 control and regulation. However, if the stirrer interferes also with the control cascade for pO_2 and pCO_2, the effect of energy transfer has to be studied in an analogous approach according to the experimental set-up shown herein. In studies of this kind, the procedural parameter always have to be considered separately from the effect of the composition of the culture media.

Since direct effects of pCO_2 control on lactate metabolism could be identified, a cell line optimized with regard to lactate metabolism by means of metabolic engineering is used herein. The cell line CHO-hGM-CSF-PYC2 has a pyruvate carboxilase with cytosolic activity affecting also the central metabolism (Wagner, 1998; Irani, 1999; Bollati Fogolin, 2001; Bollati Fogolin, 2003) (FIG. **37**). pCO_2 control should allow, for the first time, to purposefully change the hydrogen carbonate concentration in the cell and, thus, the concentra-

tion of a substrate of pyruvate carboxylase via the pCO_2 level in the medium and thus influence the central metabolism.

Subsequently, the effect of different controlled pCO_2 concentrations on parameters such as cell physiology and cell metabolism as well as on intracellular pH and productivity was to be studied on a 1 L-scale using the fed-batch fermentation of cell line CHO-hGM-CSF-PYC2 which secretes the growth factor hGM-CSF into the medium. Constant cultivation conditions which are analogous to the 10 L-scale allow to compare the results with the results obtained for the antibody producing cell line CHO-MUC1. The pyruvate carboxylase from *S. cerevisiae* which exhibits cytosolic activity in this recombinant cell line and which catalyses the anaplerotic reaction of pyruvate with hydrogen carbonate into oxalacetate allows the metabolic exploitation of hydrogen carbonate, which is present in the cytosol, by the cell via the citric acid cycle (FIG. **37***b*).

According to the analysis of the metabolic flows in BHK-PYC cells expressing the recombinant cytosolic pyruvate carboxilase (Paul, 2006), the flow of the recombinant cytolsolic pyruvate carboxylase was very low under the process conditions examined in this context. Consequently, a dependency on pCO_2 concentration and a pH_i dependency of this metabolic pathway caused by said concentration dependency were to be studied. This approach was considered purposeful since the hydrogen carbonate concentration is an important parameter for the reaction of cytosolic pyruvate into oxalacetate and since, according to the results obtained herein, it should be possible to influence hydrogen carbonate concentration via the (controlled) pCO_2 level in the culture medium (and, thus, also in the cytosol). It is also conceivable to influence the newly introduced metabolic pathway in the model cell line CHO-hGM-CSF-PYC2 via the secondary parameter pH_i—which, also according to the results shown herein, can be influenced by the pCO_2 level—, for example, via the pH dependency of pyruvate carboxylase activity and lactate dehydrogenase activity.

(A) Process Conditions

Dependency of the cellular parameters on the controlled pCO_2 level was studied. The inoculum was cultivated at 5% CO_2 in the incubator as described above. All inoculum cultures used had viabilities higher than 95%. In the 1 L stirred reactor, standard fermentation conditions were used (Example 1(E)). Glucose concentration was maintained in the range of 2-5 gL^{-1} by adding a sterile bolus (maximum 5% v/v; 1-2d^{-1}) of a concentrated feed solution. This allowed to prevent both, glucose limitation and the Crabtree effect (also: negative Pasteur effect) which occurs with a glucose concentration of approximately 10 gL^{-1} and higher. Accordingly, the glutamine concentration was maintained between 0.5-1.0 gL^{-1} and amino acid limitations were avoided. Throughout the entire cultivation process, pCO_2 values were controlled to be constant 5% pCO_2 and 15% pCO_2, respectively. A cultivation with uncontrolled pCO_2 concentration was run as reference (FIG. **38**). Here, pH set value deflections of the culture medium were counteracted by adjusted admixture of CO_2 in the gassing air as well as by controlled addition of sodium carbonate solution (1 M). With this CO_2-based pH control, the increased formation of acidic metabolites (e.g. lactate) during the cultivation process results in a reduction of the CO_2 ratio in the supply air but it may cause a decrease of the pCO_2 concentration in the medium below physiological concentrations (<5%). This reduction can already be observed with the set-up with constant gassing rate as used herein (FIG. **38**) and is probably further increased upon successive increase in the gassing rate which is used in order to increase the oxygen transfer into the culture medium.

(B) Viable Cell Densities and Viability

Viable cell densities and viabilities are shown in FIG. **39**. In this context, the following tendencies can be observed. All cultures reach the stationary growth phase after approximately the same time span (170 h; 7 days). For the different pCO_2 profiles, the maximum viable cell densities do not differ significantly (9-11×10^6 mL^{-1}). The later entry into the stationary phase with controlled pCO_2 of 15% may be caused by the initially lower viability of the culture subsequent to inoculation of the stirring vessel. Possibly, the transition of the cells from the pre-cultivation at 5% CO_2 into the culture medium equilibrated at 15% CO_2 in the reactor first has a negative effect on the viability. Effects of these pCO_2 surges on the cells were studied in Example 4. It proved that, in comparison with the process according to the state of the art in which pCO_2 is not controlled, the control of pCO_2 concentrations throughout the whole fermentation process has a positive effect on the viability of the cultures and allows an extended stationary phase of high cell density. The higher the controlled pCO_2 value, the longer the culture is viable. The optimum for the recombinant CHO-K1 cell line studied is closer to 15% pCO_2 than to 5% pCO_2.

(C) Addition of Base and Osmolalities

The adjustment of the initial controlled pCO_2 value upon inoculation was carried out with simultaneous control of the pH value. Thus, the pH value decrease due to the CO_2 added for high pCO_2 values was counteracted by the addition of sodium carbonate solution. This caused a higher initial addition of base into the fermenter at 15% pCO_2 as can be seen in FIG. **40**. However, the process without pCO_2 control initially does not require any base since the acidification of the medium can first be counteracted by a reduction of the CO_2 ratio in the supply air (FIG. **40**). Whereas the addition of base at controlled 5% pCO_2 and controlled 15% pCO_2 is identical for the intervals of the process time 80-180 h (parallel curves), the addition of base in processes with uncontrolled pCO_2 is greatly increased during this time. Thus, only the process at 15% pCO_2 requires a base until the process is terminated (FIG. **40**), implying that the metabolism of the cultivated cells is still active. The final osmolalities of all described processes are, however, identical and show an analogous curve throughout the process period (FIG. **40**). The extended process duration due to higher viabilities is, thus, not coupled to osmolality, but has to be primarily attributed to the controlled pCO_2 level.

(D) Lactate Metabolism

Also with the lactate formation of the different processes, graduation can be observed (FIG. **41**). The non-controlled pCO_2 process forms more lactate from the start of the process already and achieves, compared to the other processes, a higher concentration maximum. Subsequently, there is the controlled 5% pCO_2 process involving decreasing lactate accumulation, followed by the controlled 15% pCO_2 process.

Independently from the controlled glucose and glutamine concentrations, respectively, which were all maintained between 2.0 to 5.0 gL^{-1} and 0.5 to 1.0 gL^{-1}, respectively, over the entire fed-batch process (data not shown), in the processes with uncontrolled pCO_2 and with controlled 5% pCO_2 a lactate metabolisation with entry into the stationary phase can be observed (from 180 h and 220 h, respectively, FIG. **42**). This diauxy behaviour is not very distinct in the controlled 15% pCO_2 process. Its cell-specific lactate formation rate is in the positive range over the entire course of cultivation (FIG. **42**), however, the lactate accumulation in this process is the lowest (FIG. **41**).

These observations allow for the following conclusions: In the studied fed-batch process, the cell line CHO-hGM-CSF-

PYC2 used showed lower maximum lactate concentrations with controlled pCO_2 values (reduction by 30% with 15% pCO_2 compared to non-controlled pCO_2 process). The remetabolisation of lactate observed with entry into the stationary phase is more distinct with higher lactate concentrations. This change in metabolism is not coupled to a limitation of glucose or glutamine. The central metabolism, in this case lactate formation serves as an example, can thus be influenced by control of pCO_2. In this recombinant cell system with cytosolic pyruvate carboxylase, an increase in metabolic efficiency can be achieved by increase in pCO_2 in the culture medium (preferably carried out by an appropriate control and regulation, as shown herein).

(E) Product Formation

The specific product formation rate differs with different, controlled pCO_2 values significantly (FIG. **43**). In comparison with the uncontrolled pCO_2 process, the specific product formation rates are strongly increased with continuously controlled pCO_2: for the controlled 15% pCO_2 process compared to the controlled 5% pCO_2 process by 15% on average (FIG. **43**). Thus, by control of pCO_2 up to constant 15% pCO_2, the increased specific product formation rate and the extended process duration result in a maximisation of the hGM-CSF product titre by 100% in the case of the cell line CHO-hGM-CSF-PYC2 used (FIG. **44**).

(F) pH_i Cell Cycle Phase Distribution and Specific Productivity

In the following, the cause of the increased productivity with increased controlled pCO_2 concentrations was to be analysed. For this purpose, the intracellular pH curves, specific productivities and selected cell cycle phase distributions of the individual fermentations with different pCO_2 profiles were contrasted.

In Example 4 it was clearly shown which effects pCO_2 has on pH_i. Thus, pCO_2 jumps in the culture medium of the processes always cause a biphasic change of the intracellular pH value. In the following figures (FIG. **45-47**), the curves of the intracellular pH value (before pulsed feed), the specific productivities and the ratios of the percentages of cells in the S-phase and cells in the G0G1 phase (S/G0G1) are shown.

In the fed-batch cultivation with uncontrolled pCO_2 (FIG. **45**), the first feeding coincides with the decrease of the pH_i by 0.3 units. With entry into the stationary growth phase, the pH_i increases again. In comparison with the controlled pCO_2 processes, the SPR remains extremely low. The pH_i also remains on an extremely low level ($pH_i<7.1$) during the entire cultivation.

With the 5% controlled pCO_2 culture (FIG. **46**), the pH_i decreases, independently from the entry into the stationary growth phase, due to the first feeding by approximately 0.3 units. The entry into the stationary growth phase coincides with an increase in the pH_i by 0.5 units. The SPR also strongly increases in the stationary growth phase. In total, the pH_i is on a higher level than with the uncontrolled pCO_2 culture (FIG. **45**), however, it fluctuates more than with the 15% controlled pCO_2 process (FIG. **47**). Furthermore, the increase in the S/(G0G1) ratio at the end of the stationary growth phase shows an incomplete arrestation of the cell cycle.

With the controlled 15% pCO_2 culture (FIG. **47**), after the first feeding, the pH_i does not decrease—contrary to the cultivations with uncontrolled and controlled 5% pCO_2, respectively. Even after entry into the stationary growth phase, the pH_i only decreases slightly and for a short period. On the whole, with controlled 15% pCO_2, this cultivation shows the lowest variations of the pH_i values and, moreover, the highest pH_i level in the course of cultivation. Furthermore, in this context, a constant low cell cycle phase fraction S/(G0G1) can be observed from approximately 200 h, which corresponds to a successful arrestation of the cell cycle (FIG. **47**). The thus extended viable culture period in combination with the increasing SPR during the stationary growth phase results in the highest end product titre in this cultivation series (FIG. **44**).

In summary, the cell-specific productivities with the cell cycle phase fractions of the cells in the G0G1 phase are shown for the processes with different pCO_2 profiles in FIG. **48**.

Accordingly, the following results can be summarised for the described fermentations with different pCO_2 profiles for the cell line CHO-hGM-CSF-PYC2 analysed: An increasing pH_i correlates with the increased G0G1 phase fraction of cultivated cells. An increased G0G1 phase fraction correlates with an increase in the cell-specific productivity. The higher the controlled pCO_2 set value during the course of the process, the higher is the phase fraction G0G1 in the stationary growth phase.

The increase in the specific productivity by arrestation of cells in the G0/G1 phase is described in the literature. A three-fold higher specific production formation rate was observed e.g. with use of AMP (adenosine monophosphate), since the cells during the exponential growth phase were arrested and, thus, transferred into a long stationary phase.

The process strategy of pCO_2 control as pursued herein results for CHO-hGM-CSF-PYC2 in a product concentration which is 10-fold higher (at controlled 15% pCO_2) than with the strategy based on temperature decrease by Bollati et al. (Bollati Fogolin, 2003). According to the findings obtained therein, it can be assumed that also in the latter case solubility of CO_2 in the culture medium was increased due to the temperature decrease and, thus, may have played a role in the increased productivity via an extended process duration and an extended G0/G1 phase. Similar effects due to temperature decrease have already been described (Bloemkolk, 1992; Moore, 1997; Kaufmann, 1999). Even tough, contrary to the batch method, in the fed-batch method used, limitations of the substrate can of course be avoided and an extended process duration can be achieved, increased, controlled pCO_2 is a basic factor for higher specific product formation rates. This increase is probably related to the increased solubility of CO_2 in the culture medium.

(G) Cell Respiration

In combination with pH control, measurement and control of pCO_2 in the culture medium developed in the present invention an enrichment of CO_2 in the liquid phase was counteracted (Example 2). Thus, it became possible to balance CO_2 by means of supply and exhaust air. The correlation of the oxygen uptake rate (OUR) with the carbon dioxide evolution rate (CER) results in the respiratory quotient (RQ). A complete respiration of glucose into CO_2 would result in RQ=1. An RQ value >1 would only occur with fermentation, lipid or protein anabolism, whereas an RQ value <1 is an indicator for incomplete oxidation of amino acids and for protein and lipid catabolism (Hauser and Wagner, 1997; Alberts, 2002).

As described in Example 1, the oxygen transfer rate OTR can be calculated based on the k_La value. This value was determined cell-free under standard pressure in culture medium using the reconcentration method under standard fermentation conditions in both reactors. For the 1 L reactor used under atmospheric pressure, $k_La_{O2}=3.55\ h^{-1}$. Using the diffusivities of the gases oxygen and carbon dioxide via the proportionality factor 0.89 (Frahm, Blank et al., 2002), the value for this reactor is $k_La_{cO2}=3.16\ h^{-1}$.

The OUR was calculated using the flow volume of oxygen in the supply air. The partial pressure of oxygen in the reactor

was measured online by means of an oxygen sensor. Based on the pO_2 value, the concentration of oxygen dissolved was calculated using the Henry constant for oxygen at 37° C. Based on the oxygen concentration in the supply air, the theoretical saturation concentration c* was calculated. This allowed to calculate the OTR value. In a stationary controlled state, OTR is equal to OUR and CTR is equal to CER, respectively.

In analogy to OUR, CER can be calculated on the basis of the measurement of the exhaust air. FIGS. **49** and **50** show the OUR, CER and RQ values for the pCO_2 controlled processes. After reaching a maximum value, the RQ value shows a substantially more rapid decrease at 5% pCO_2 than in the controlled 15% pCO_2 process. After a process time of 300 h, the RQ value of the process at 5% pCO_2 is merely a third of the RQ value at 15% pCO_2. The metabolism of this comparatively more productive process with controlled 15% pCO_2 is more oxidative throughout the whole process duration than the metabolism of the process with controlled 5% pCO_2.

Due to the comparatively high gas volume flows, the low cell consumption and production rates allow no balancing with respect to the differences in concentrations in supply and exhaust air. In this respect, the RQ calculations presented herein have some disadvantages in comparison with calculations described for microbial cultivations. Nonetheless, the RQ calculations demonstrate the potential of pCO_2 control to determine the parameter RQ in real time during fermentations also in animal cell cultivation processes. This would allow RQ parameter based process control in industrial high cell density processes via pCO_2 and, consequently, via purposeful adjustment of pH_i with the above-mentioned optimization potentials.

The product formation occurs essentially in the G0G1 phase of the cell line studied. The higher the controlled pCO_2, the higher is the number of cells remaining in this phase. Thus, the higher viability of cells at a higher controlled pCO_2 implies that cells remain longer in the G0G1 phase with an increased pCO_2.

(H) Apoptosis

It was studied whether a higher controlled pCO_2 level has an anti-apoptotic effect on the cell culture. The 5-phase pH_i curve is a common characteristic of all cultivations described above, which is clear from FIGS. **45** to **47**. It is highly probably that, in all cultivations, the last turning point of the pH_i curve marks the beginning apoptosis. In this context, the cell alkalinizes intracellularly before viabilities decrease below 80%, which was the criterion for stopping all cultivations. In the flow cytometric measurements of cell cycle which accompanied cultivation, a higher number of DNA fragments was also detected as sub-G1-peak after this late pH_i turning point (data not shown) which in general can be observed in late phases of apoptosis (budding).

(I) Consequences for Process Development

With uncontrolled pCO_2 or pCO_2 controlled at a low level (5%), pulsed feeds can have unfavourable effects on the intracellular pH value (FIG. **45**-**47**). Apart from this feed technique, the pharmaceutical industry uses continuous feed throughout the whole fed-batch process in analogous processes. If pCO_2 is not controlled to maintain a relatively constant and/or high level, respectively (see above), this is a possibility not to provoke uncontrolled pH_i oscillations based on the results shown herein to begin with. It would, however, be possible to purposefully achieve the same effect on the pHi of cells and, thus, on their physiology and metabolism by combining pulsed and continuous feed, which would have to optimized, preferably in combination with pCO_2-controlled cultivation. A strategy adapted to the process phases of growth and production might be rationally optimized on the basis of the findings of the present invention. Due to the high intracellular buffer capacity by HCO_3^-, a high (controlled) pCO_2 content in the medium causes lower environment-dependent pH_i oscillations.

ABBREVIATIONS

Abbreviation	Explanation
ATCC	American Type Culture Collection
BA	butyric acid
BGA	blood gas analyzer
BHK	baby hamster kidney
BSA	bovine serum albumin
CA	carboanhydrase
CD	chemically defined
CER	CO_2 evolution rate
CHO	Chinese hamster ovary
CIP	clean-in-place
CMV	cytomegalovirus
CTR	CO_2 transfer rate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPN	pressure-controlled sampling apparatus
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-assisted cell sorting
FBS	fetal bovine serum
FL	fluorescent light
FSC	forward scatter
G418	genicitin
GFP	green fluorescent protein
GLC	glucose
GLN	glutamine
GLT	glutamate
HDFSB	dialysed FBS in HEPES buffer
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
hGM-CSF	human granulocyte-macrophage colony-stimulating factor
HPLC	high performance liquid chromatography
HPTS	hydroxypyrene-3-sulfonic acid
HRP	horseradish peroxidase
HTS	high throughput screening
IgG	immunoglobulin
LAC	lactate
LGH	lactate dehydrogenase
MCB	master cell bank
MFC	mass flow controller
MUC1	mucin glycoprotein
NAD	nicotinamide adenine dinucleotide
NHE	sodium/proton exchanger
OPA	ortho-phthaldialdehyde
OPD	1,2-phenylenediamine dihydrochloride
OSM	osmolality
OTR	oxygen transfer rate
OUR	oxygen uptake rate
PBS	phosphate buffered saline
pH_i	intracellular pH
pNPP	p-nitrophenol phosphate
PRO	product
PYC	pyruvate carboxylase
RNA	ribonucleic acid
RP	reverse phase
rpm	revolutions per minute
RQ	respiratory quotient
RZA	space-time yield
SNARF-1	5'(and 6')-carboxy-10-dimethylamino-3-hydroxyspiro[7H-benzo[c]xanthene-7,1'(3'H)-isobenzofuran]-3'one
SSC	sideward scatter
TCA	tricarboxilic acid cycle, citric acid cycle, citrate cycle
TMA	trimethylamine
tPA	tissue plasminogene activator
VIA	viability

-continued

Abbreviation	Explanation
WCB	working cell bank
WTR	growth rate
ZDG	total cell density
ZDL	viable cell density

REFERENCES

Alberts, B. J. et al. (2002). *Molecular Biology of the cell*, Garland Science.
Bailey, J. and D. 011 is (1986). *Biochemical Engineering Fundamentals*, McGraw-Hill.
BD_Biosciences (2000) Manual Part Number 11-11032-01.
Bell S L, et al. (1995) Enzyme Microb Technol 17: 98
Bloemkolk, J. et al. (1992) *Biotechnol Bioeng* 40:427-431.
Bollati Fogolin, M., et al. (2002) *Electron. J. Biotechnol.*
Bollati Fogolin, et al. (2001) Proceeding of the 17th ESACT Meeting, Tylösand, Kluwer Academic Publishers, Dordrecht, The Netherlands.
Bollati Fogolin, M., et al. (2003) *J. Biotechnol.* 109 (2004): 179-191.
Bond, J. and J. Varley (2005). *Cytometry* Part A (64A): 43-50.
Bresnahan, W. A., et al. (1996) *Virology* 224: 150-160.
Büntemeyer, H. (1988) Entwicklung eines Perfusionsprozesses zur kontinuierlichen Kultivierung tierischer Zellen in Suspension. Hannover, Universität Hannover.
Büntemeyer, H., et al. (1991). *Cytotechnol.* 5: 57-67.
Chen, K., et al. (2001) Biotechnol. Bioeng. 72:55
Cherlet, M., et al. (1999) *Biotechnol Prog* 15(4): 630-9.
Chow, S. and D. Hedley (1997) *Current Protocols in Flow Cytometry,* 9.3.1-9.3.10, John Wiley & Sons, Inc.
Chow, S., et al. (1996) *Cytometry* 24(4): 360-7.
deZengotita, V. M., et al. (2002) *Biotechnol. Bioeng.* 77(4): 369-380.
Dittmer, D. and E. S. Mocarski (1997) *J. Virol.* 71: 1629-1634.
Engasser, J. M., et al. (1996) *Flow cytometry applications in cell culture*. M. Al-Rubeai and A. N. Emery. New York, Marcel Dekker.
Etcheverrigaray, M., (1998) *Animal Cell Technology: New Developments, New Applications*. J. B. Griffiths, Merten, O. W. (Ed.), Kluwer Academic Publishers, Dordrecht, The Netherlands.
Frahm, B., et al. (2002) *J Biotechnol* 99(2): 133-48.
Givan, A. L. (1992). *Flow Cytometry: First Principles* New York, Wiley-Liss.
Grinstein, S., et al. (1989). *Biochim. Biophys. Acta* 988: 73-97.
Hauser, H. and R. Wagner (1997) *J Biotechnol* 59(1-2): 103-115.
Hu, W. S., et al. (2007) An advanced course in cellular bioprocess technology—Fundamentals and frontiers. Garmisch-Partenkirchen.
Irani, N., et al. (1999). *Biotechnol. Bioeng.* 66: 238-246.
Jockwer, A., et al. (2005) *Pressurisable sampling device for representative measurement of dissolved gases in large-scale fermentation processes*. Vortrag Bioperspectives 2005, Wiesbaden.
Jockwer, A., et al. (2005) DE 10 2005 020 985.8-41.
Kaufmann, H. M., (1999) *Biotechnol Bioeng* 3: 573-582.
Kim, M. S., et al. (2002) *In Vitro Cell. Dev. Biol. Animal* 38: 314-319.
Kimura, R. M. et al. (1996) *Biotechnol Bioeng* 52(1): 152-160.
Klinger, C. (2006) Diplom Thesis. Weihenstephan, University of Applied Sciences Weihenstephan.
Langheinrich, C. (1999) *Biotechnol. Bioeng.* 66(3).
Lim, C. K. (1987). HPLC of small molecules—a practical approach. Oxford, IRL Press, Oxford.
Link, T. (2003) Thesis Mathematisch-Naturwissenschaftliche Fakultät. Bonn, Rheinische Friedrich-Wilhelms-Universität Bonn.
Link, T. et al. (2004) Journal of Biotechnology 110: 51-62
Madshus, I. H. (1988) *Biochem. J.* 250:1-8.
Melamed, M: R. (1990). *Flow Cytometry and Sorting* New York, Wiley-Liss.
Moore, A. M., et al. (1997) *Cytotechnology* 23: 47-54.
Osman, J. J., et al. (2001) *Biotechnol Bioeng* 75(1): 63-73.
Osman, J. J., et al. (2002) *Biotechnol Bioeng* 79(4): 398-407.
Owen, C. S. (1991). *Anal. Biochem.* 204: 65-71.
Owen, C. S., et al. (1992) *J. Fluorescence* 2(2): 75-80.
Parker, C. (1990) *Methods Enzymol.* 182: 700-718.
Paredes C, et al. (1999) Cytotechnology 30: 85
Pattison, R. N., et al. (2000) *Biotechnol Prog* 16: 769-774.
Rake, H. (1993) Umdruck zur Vorlesung Regelungstechnik A and Ergänzungen (Regelungstechnik B). Aachen, RWTH Aachen.
Reusch, H. P., et al. (1995) *Am. J. Physiol.* 268: C147-C153.
Schmelzer, A. E., et al. (2000) *Biotechnol Bioeng* 67(2): 189-96.
Shapiro, H. (1994) *Practical Flow Cytometry. 3rd ed.* New York, Alan R. Liss.
Stucka, R., et al. (1991) *Mol. Gen. Genet.* 229: 307-315.
Thomas, J. A., et al. (1979) *Biochemistry* 18: 2210-2218.
Thomas, R. (1995) *Nature* 374(6523): 597-598.
Wagner, R., et al. (1998) Process for the improvement of the primary energy metabolism of mammalian cell lines
Welsh, J. P. and M. Al-Rubeai (1996) in *Flow Cytometry: Application in cell culture*. M. E. Al-Rubeai, A. N. New York, Marcel Dekker Inc.: 163-175.
Wu, P., et al. (1993) *Biotechnol Prog* 9(4): 374-84.
Wurm, F. M. (2004) *Nature Biotechnology* 22: 553-558.
Zhangi, J. A., et al. (1999) *Biotechnol Bioeng* 65: 182-191.
"DNA Sequences in Chromosomes II and VII Code for Pyruvate Carboxylase Isoenzymes in *Saccharomyces Cerevisiae*: Analysis of Pyruvate Carboxylase-Deficient Strains", Stucka et al., Mol Gen Genet 229, pp. 307-315, 1991.

The invention claimed is:

1. A method for the recombinant production of a polypeptide in a eukaryotic host cell modified in the citrate cycle to express a cytosolic pyruvate carboxylase, the method comprising the following steps:

(a) cultivating the eukaryotic host cell in a suitable medium under conditions which allow the expression of the polypeptide, wherein the content of dissolved CO_2 in the medium is maintained at a constant value in the range of 10% to 20% of the saturated solution of CO_2 under a given set of conditions, and wherein a base is added to adjust the pH of the medium; and

(b) recovering the polypeptide from the cell or from the medium.

2. The method of claim **1**, wherein the host cell is an animal cell.

3. The method of claim **2**, wherein the animal cell is a mammalian cell.

4. The method of claim **3**, wherein the mammalian cell is a CHO, BHK, hybridoma or myeloma cell.

5. The method of claim **1**, wherein the host cell modified in the citrate cycle is a cell which expresses a cytosolic pyruvate carboxylase.

6. The method of claim **1**, wherein the polypeptide is a fusion protein, an antibody or a fragment thereof, an interferon, cytokine or growth factor.

7. The method of claim **1**, wherein the content of dissolved CO_2 in the medium is maintained at a constant value in the range of 12.5% to 17.5% of the saturated solution of CO_2 under a given set of conditions.

8. The method of claim **1**, characterized in that it is carried out as a fed-batch method.

9. The method of claim **1**, wherein the content of dissolved CO_2 in the medium is maintained constant by means of a control system with a cascaded pCO_2-controller via mass flow controllers (MFC).

10. The method of claim **9**, wherein any increase in the content of dissolved CO_2 in the medium is first reduced by decreasing a CO_2 ratio in a supply air, and if the CO_2 ratio in the supply air is reduced to zero, and the content of dissolved CO_2 still exceeds a set value, then the cascaded controller delivers an additional amount of N_2.

11. The method of claim **1**, wherein the host cell is an insect cell.

12. The method of claim **1**, wherein the base is Na_2CO_3.

13. The method of claim **1**, wherein the content of dissolved CO_2 in the medium is maintained at a constant value of 15%.

* * * * *

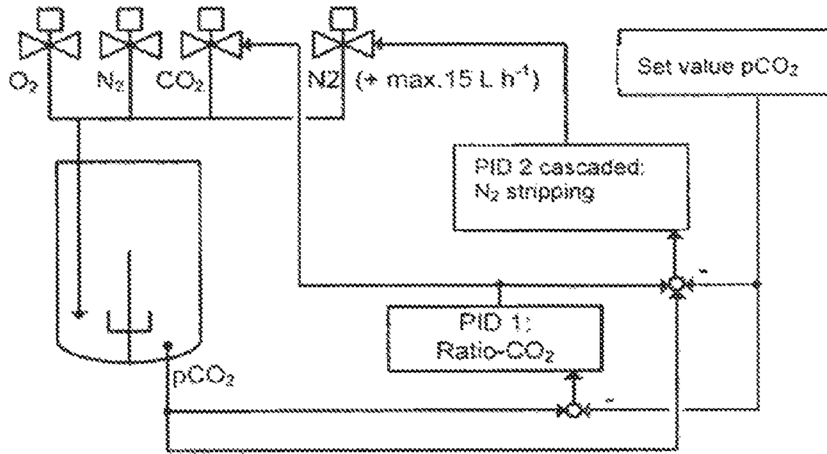
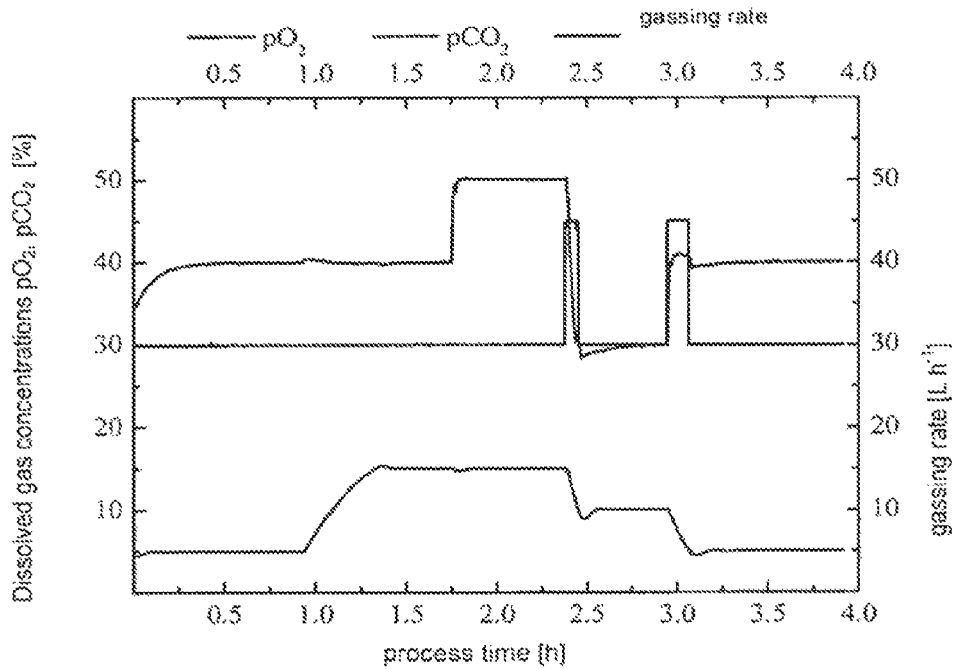


Figure 1



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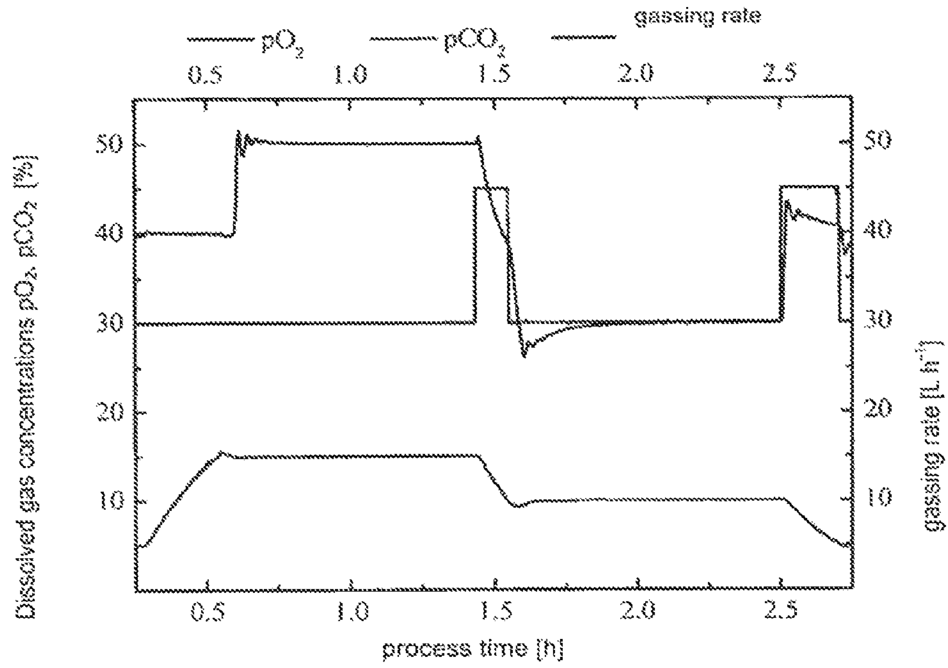

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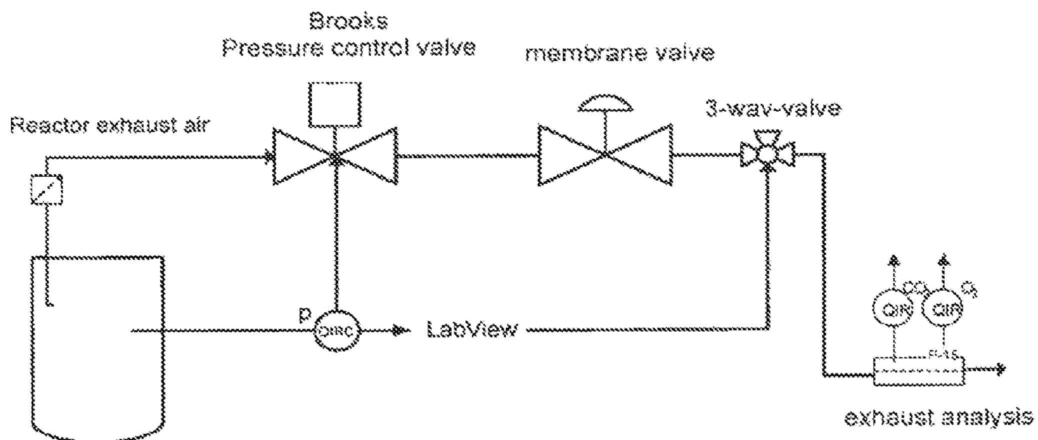

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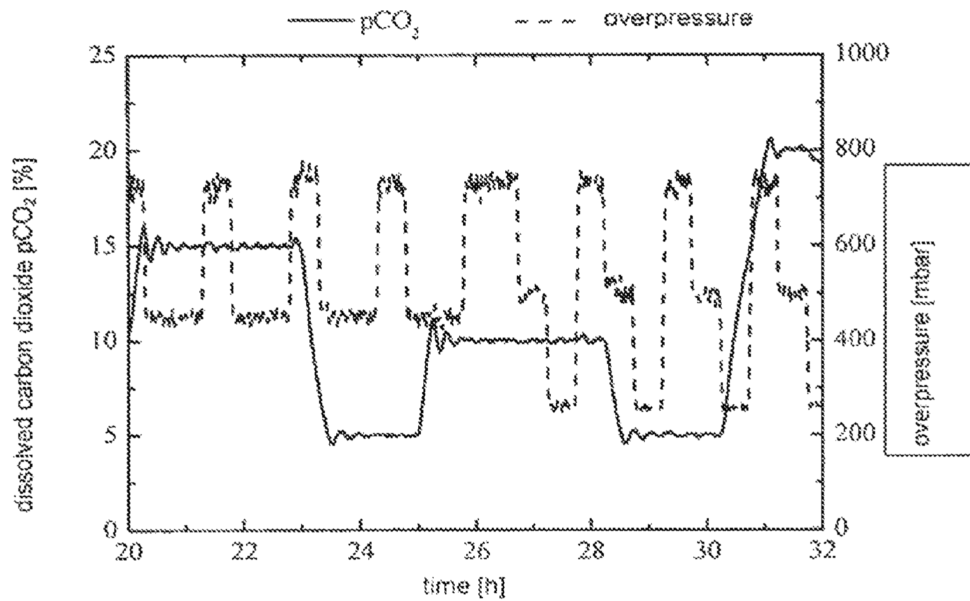

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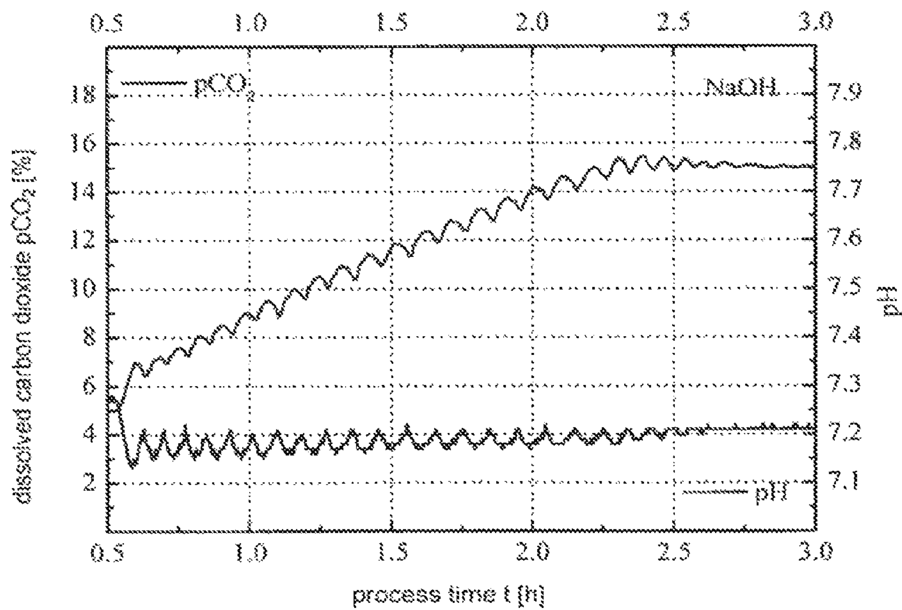

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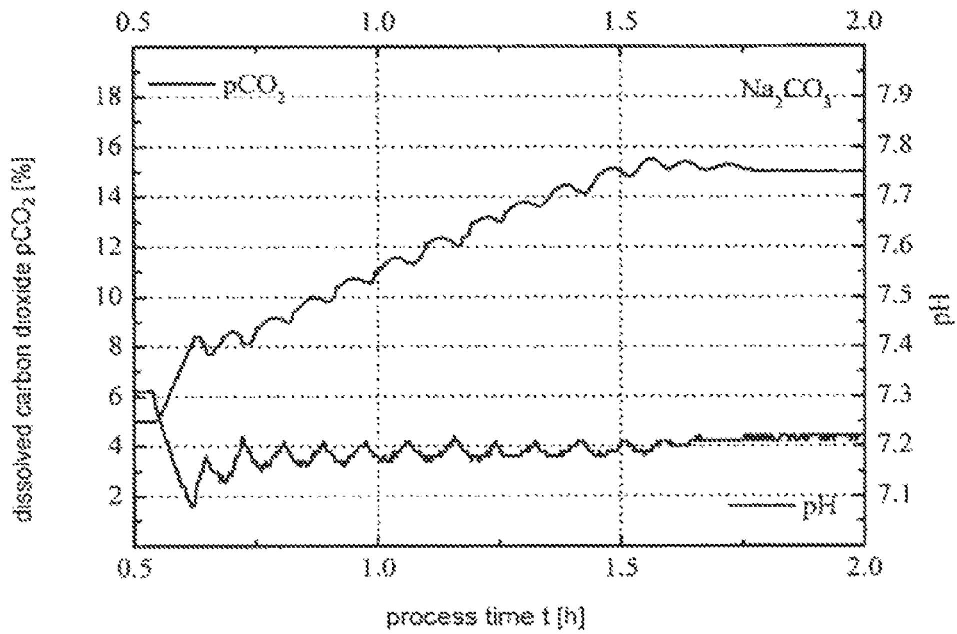

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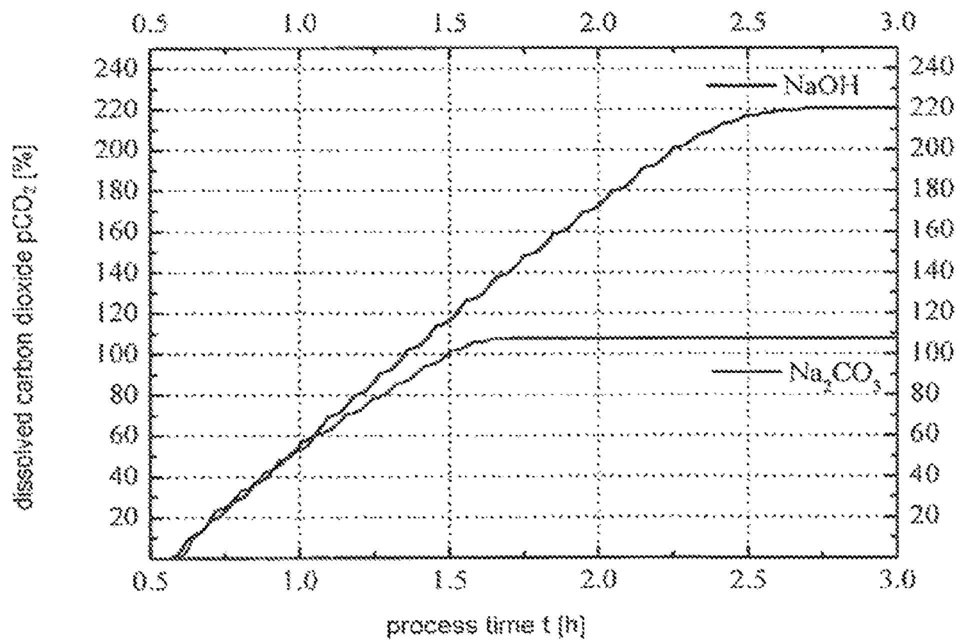

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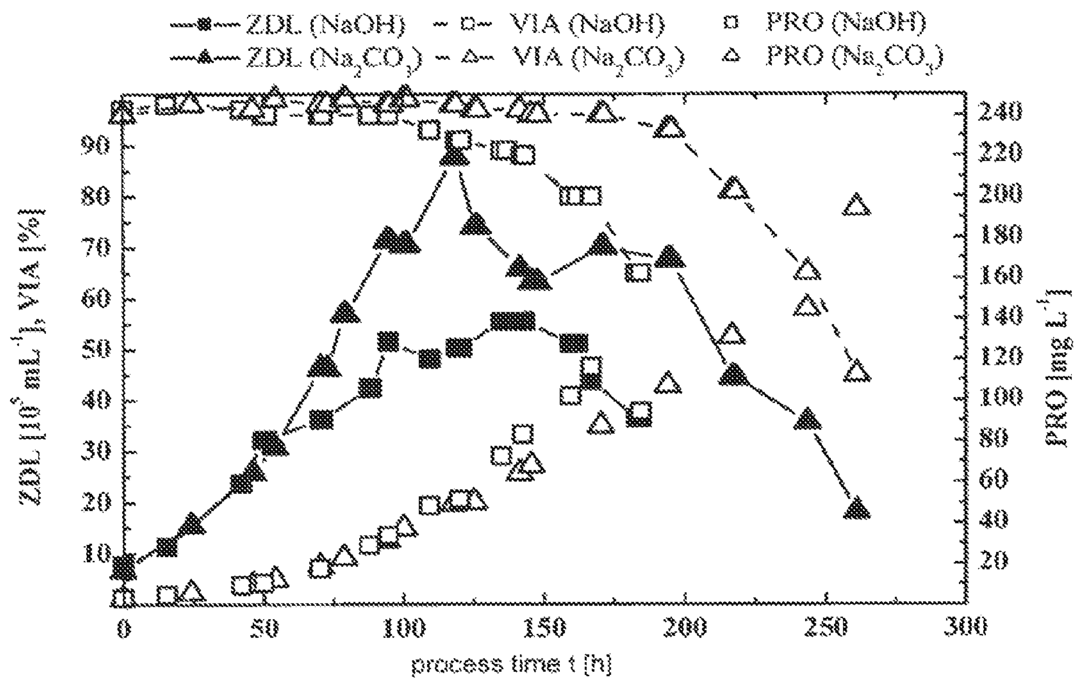

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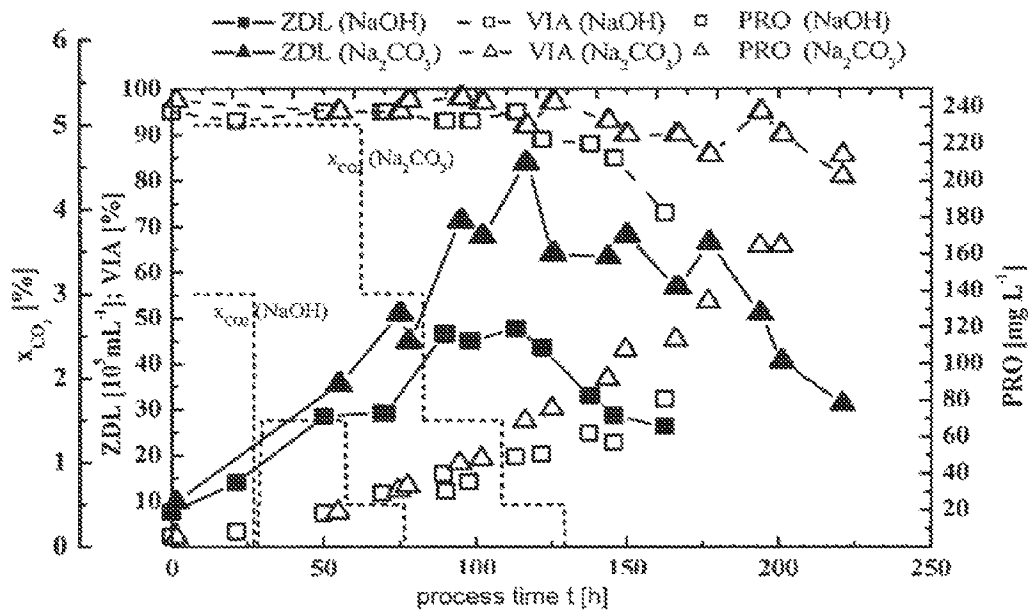

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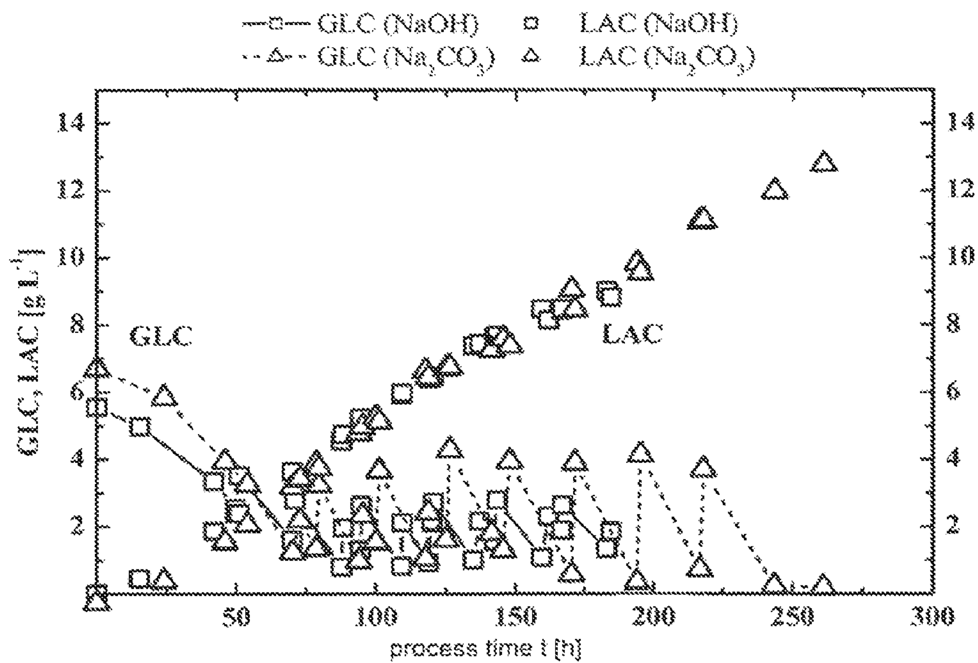

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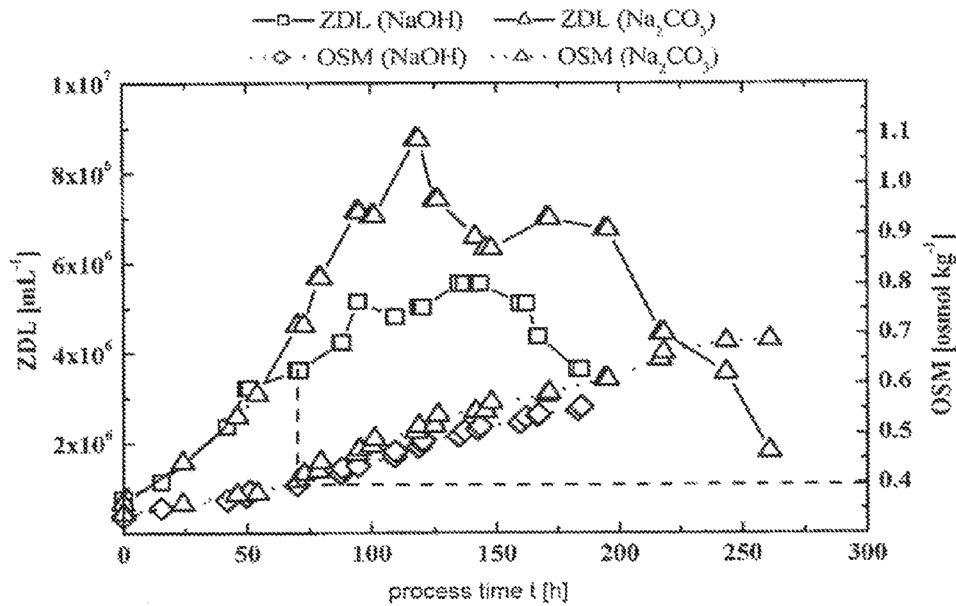

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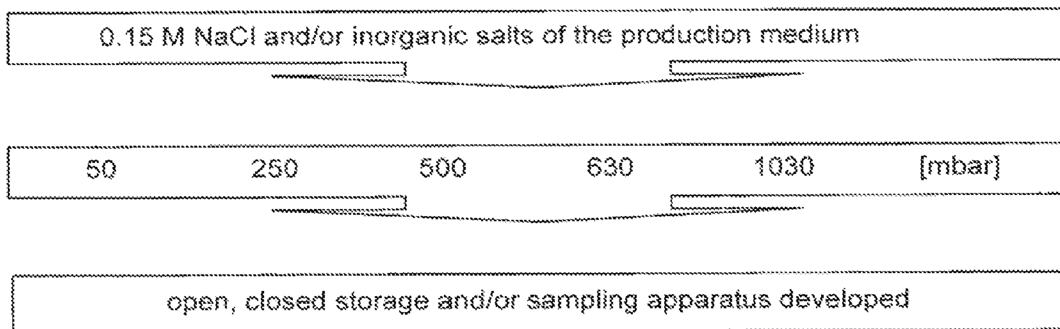

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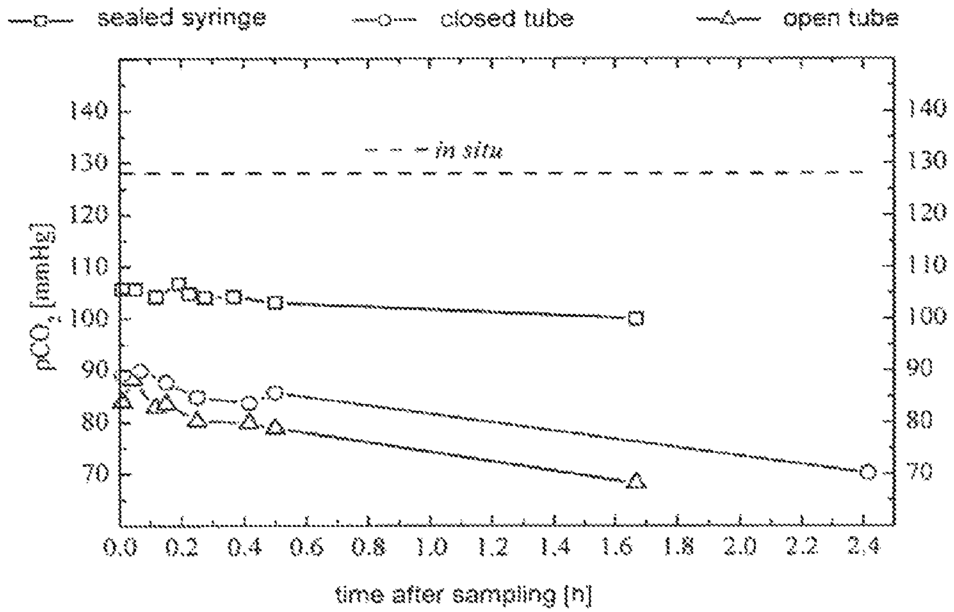

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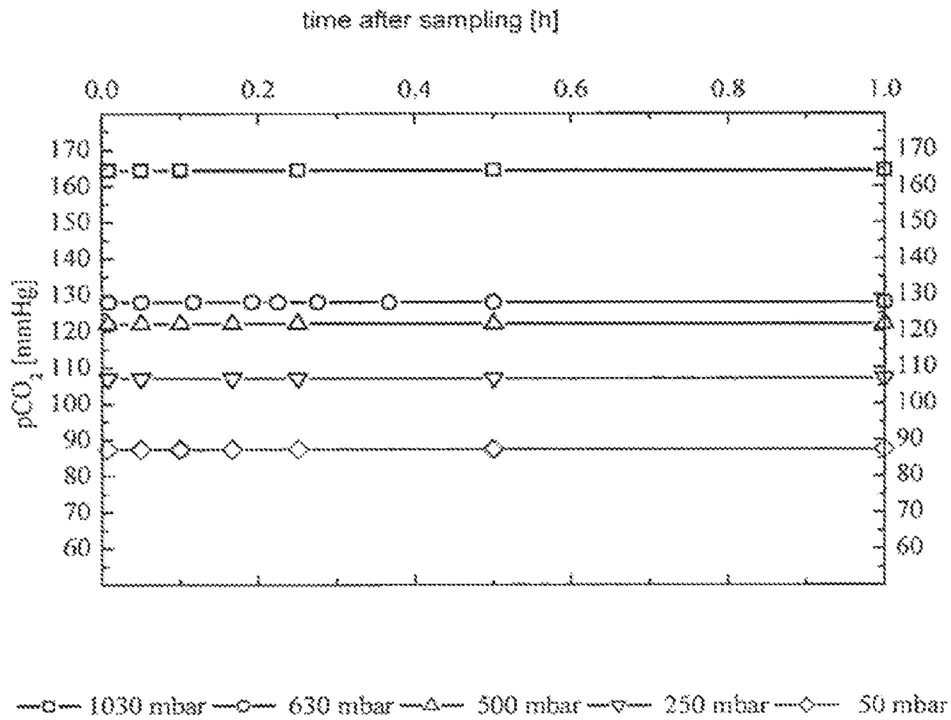

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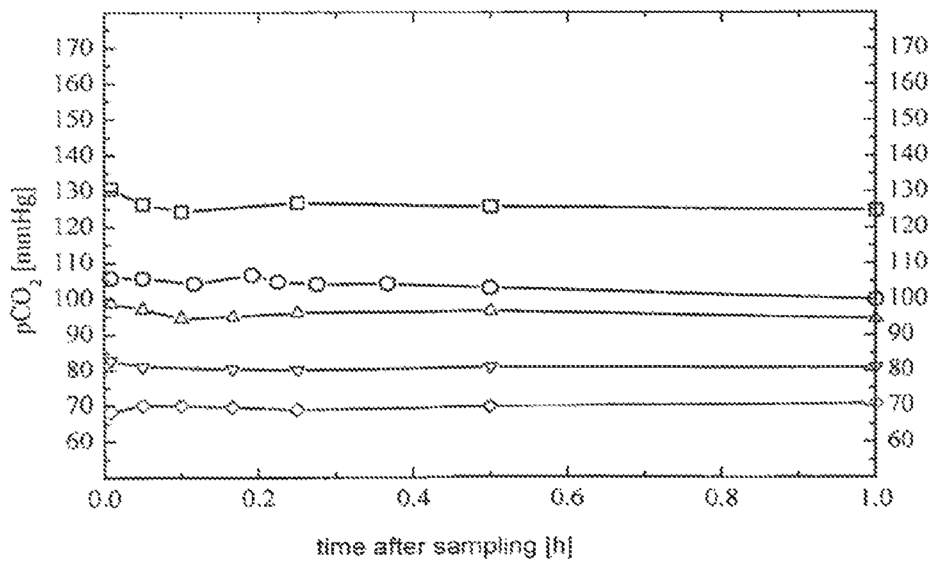

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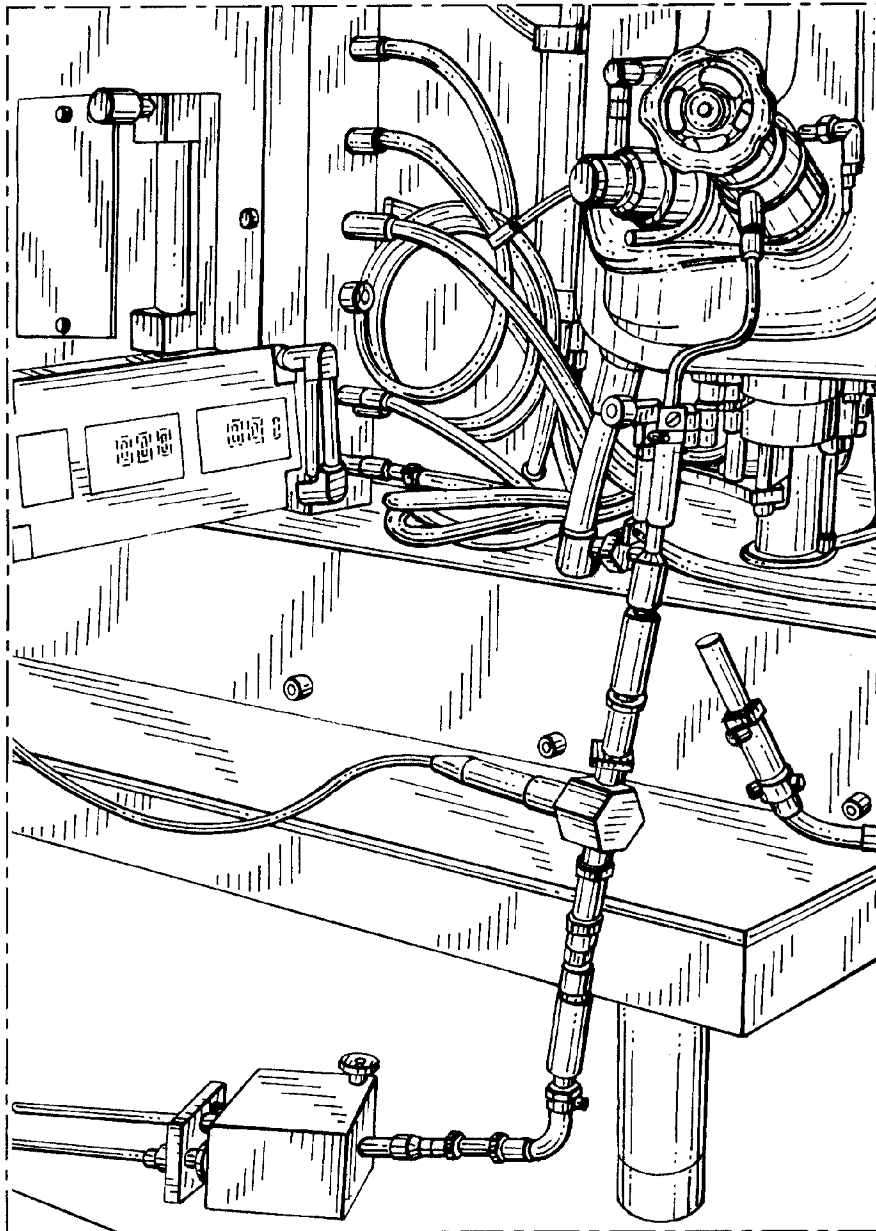

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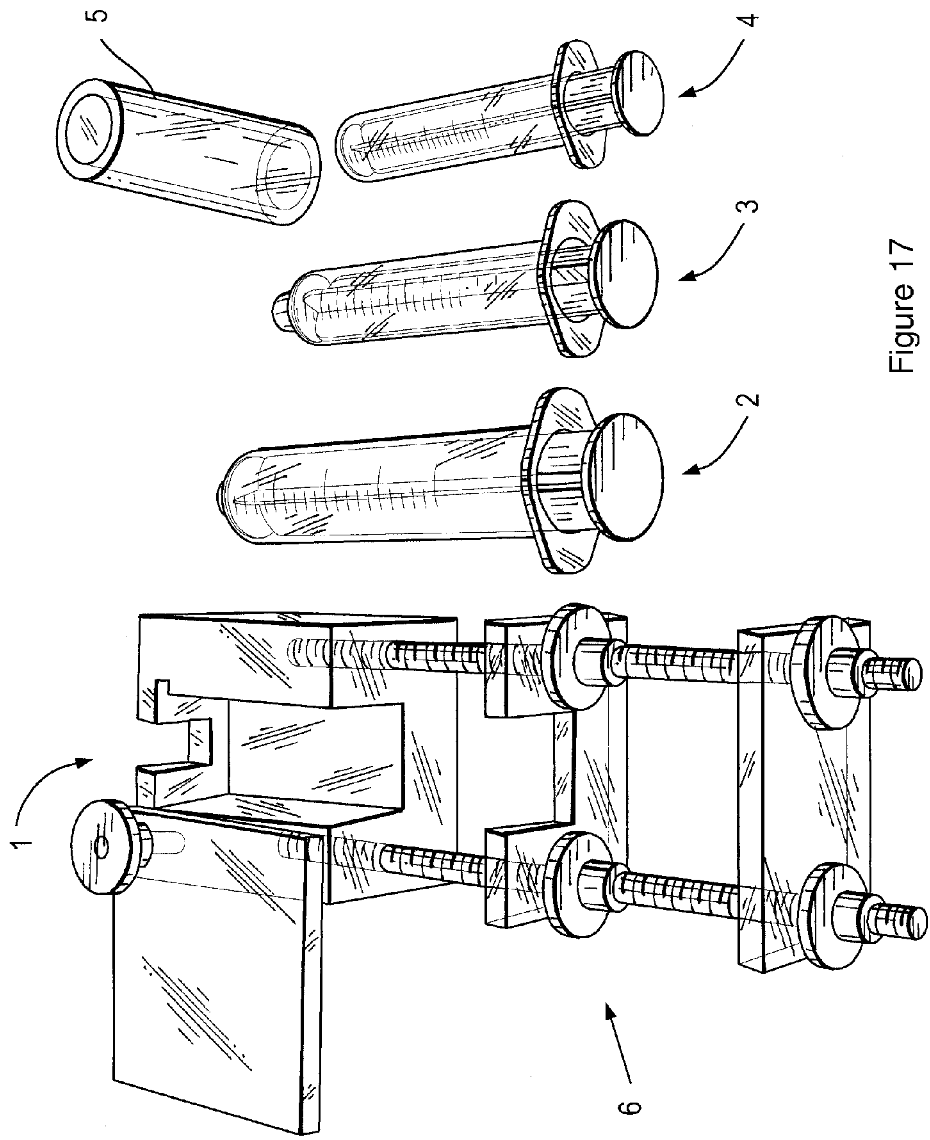

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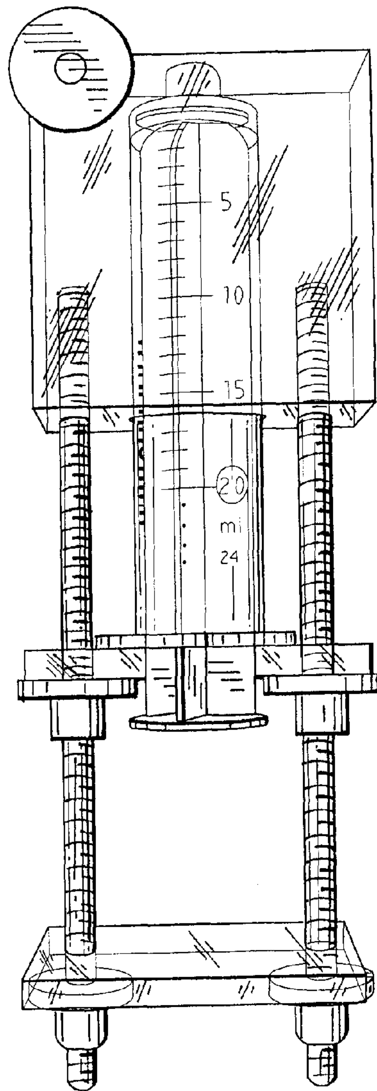

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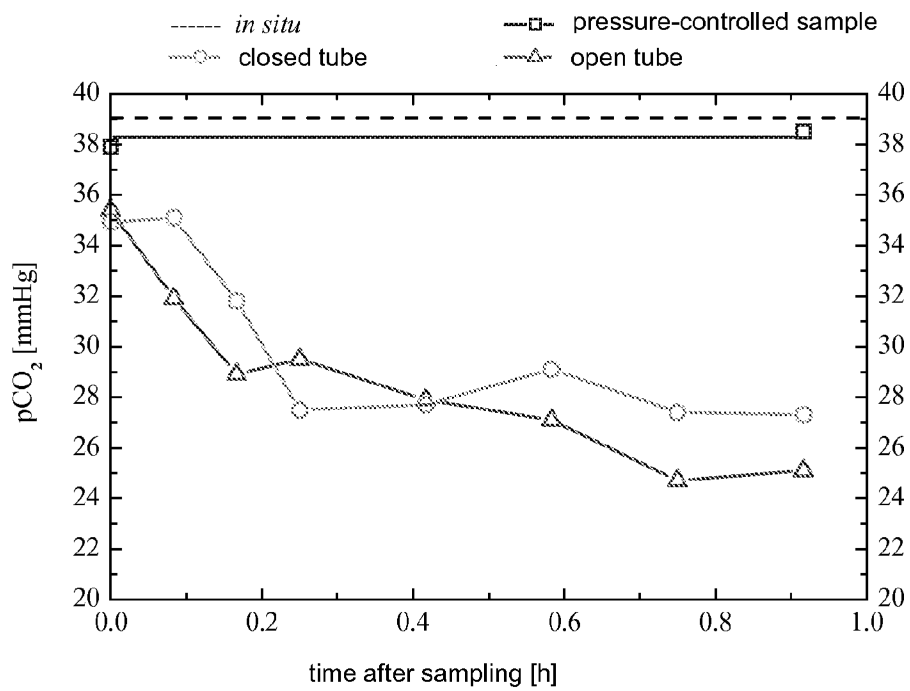

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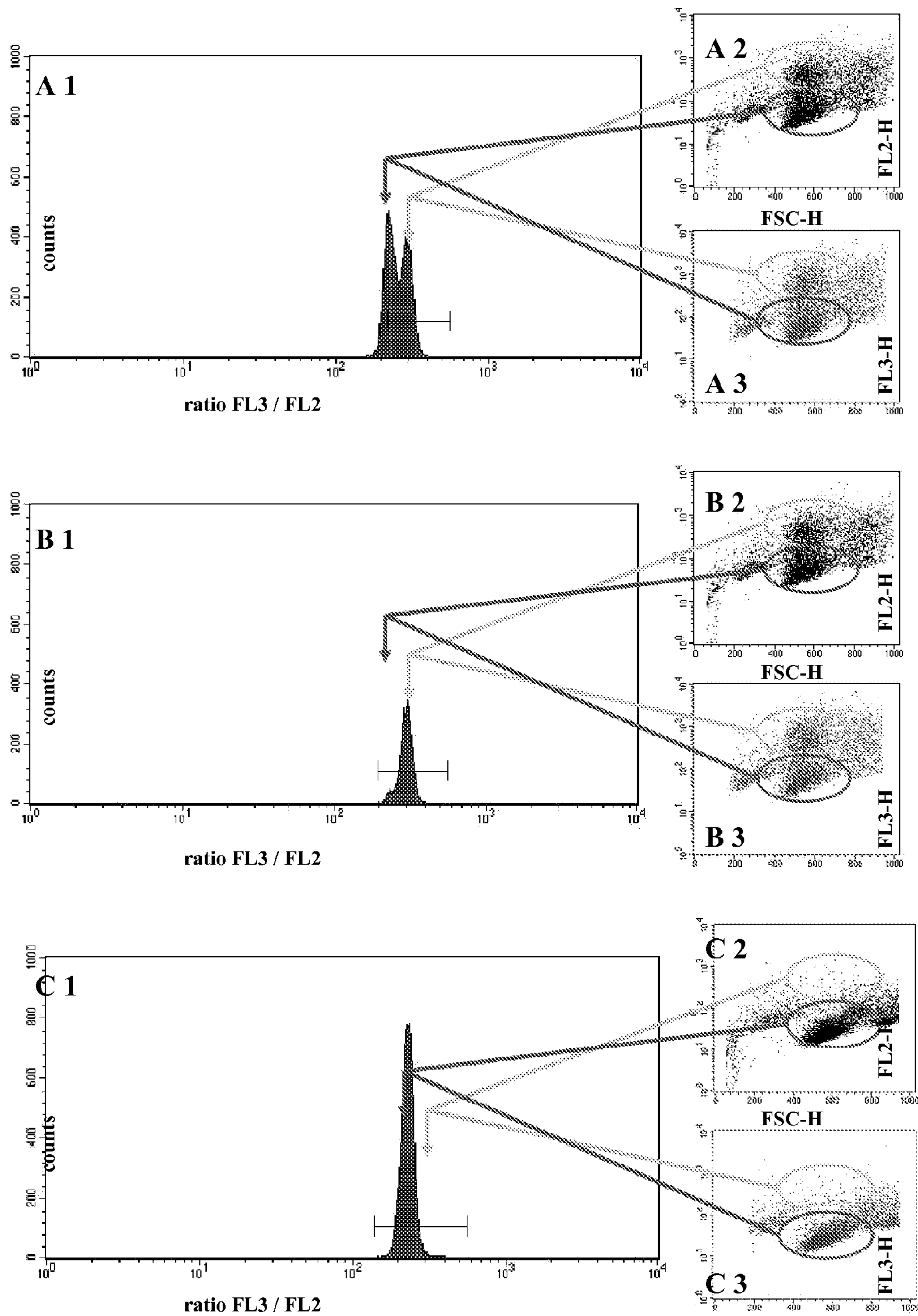

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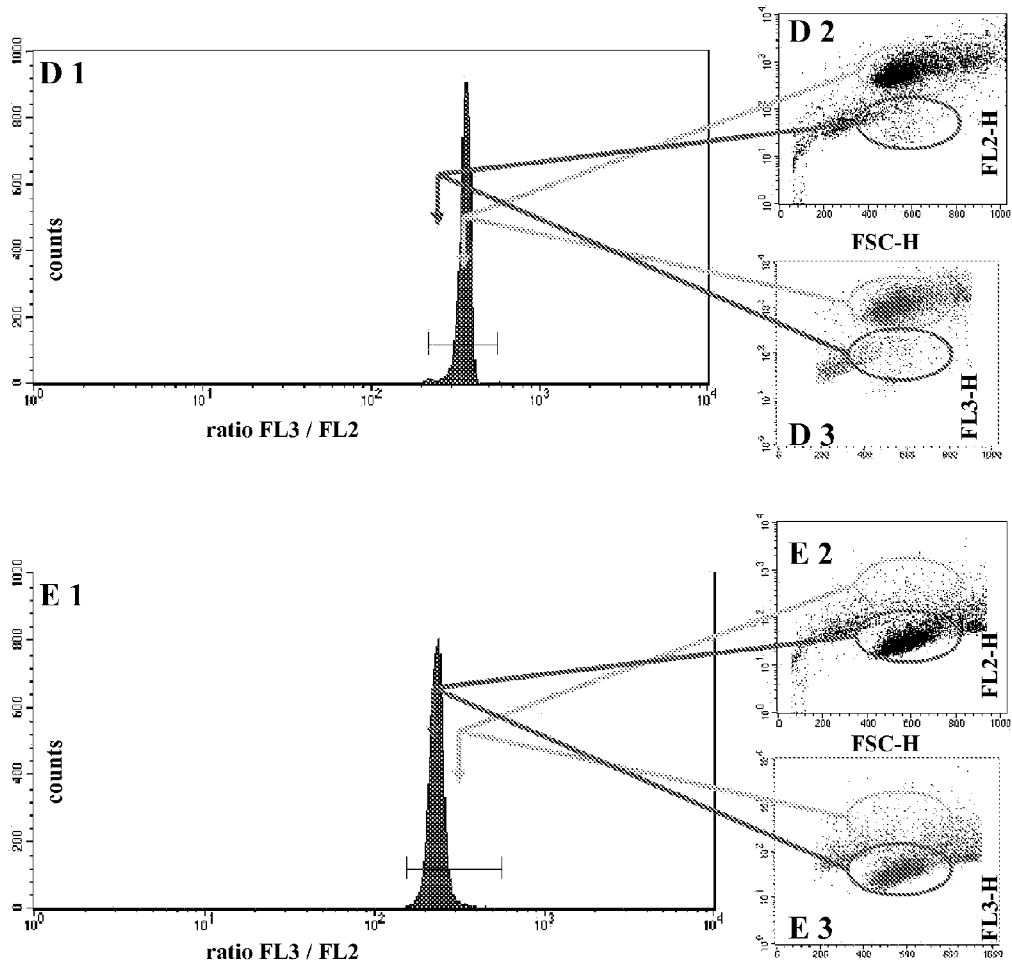

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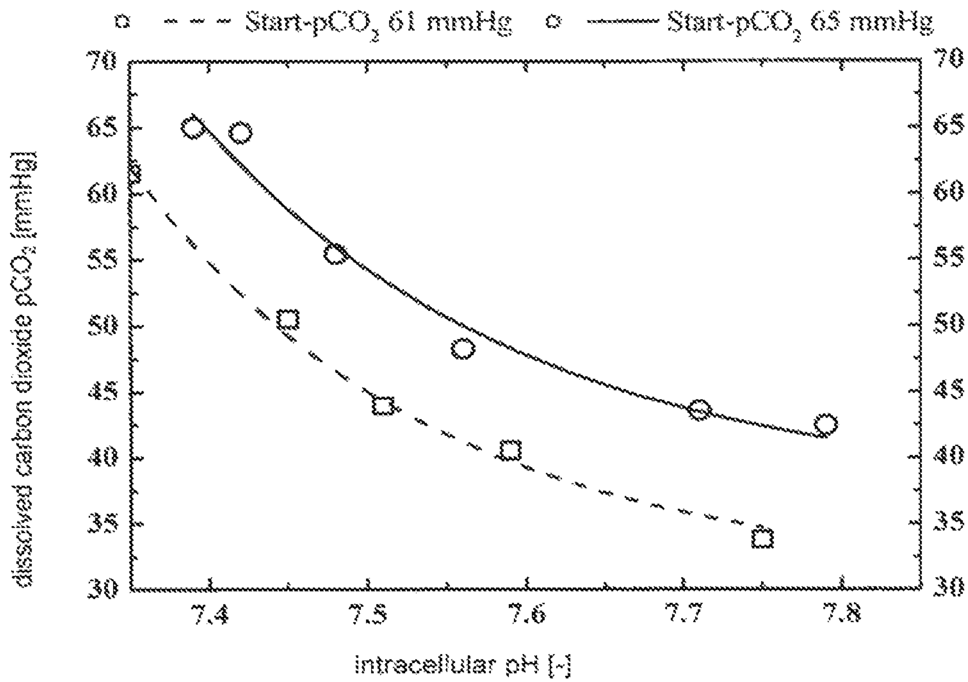

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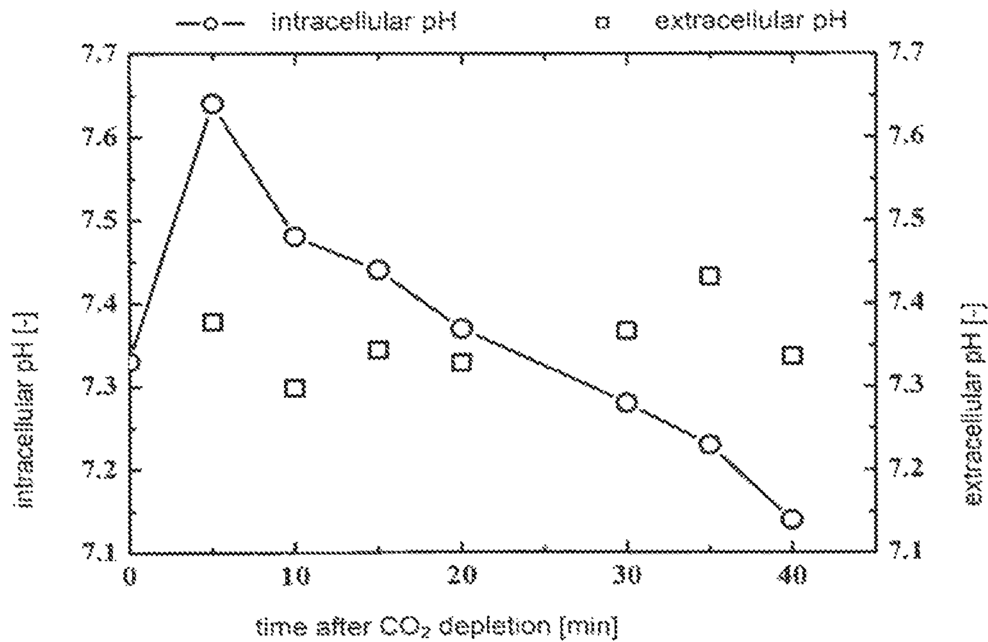

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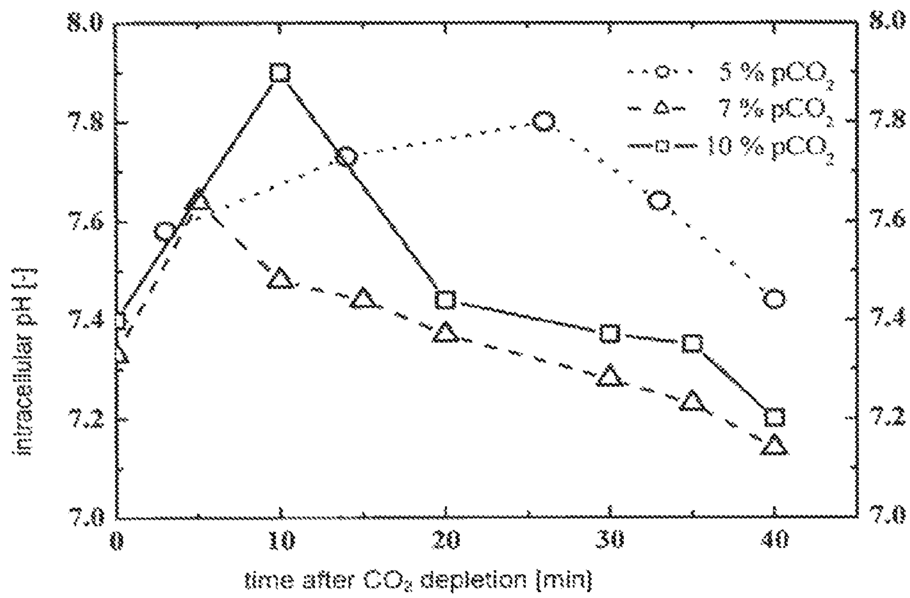

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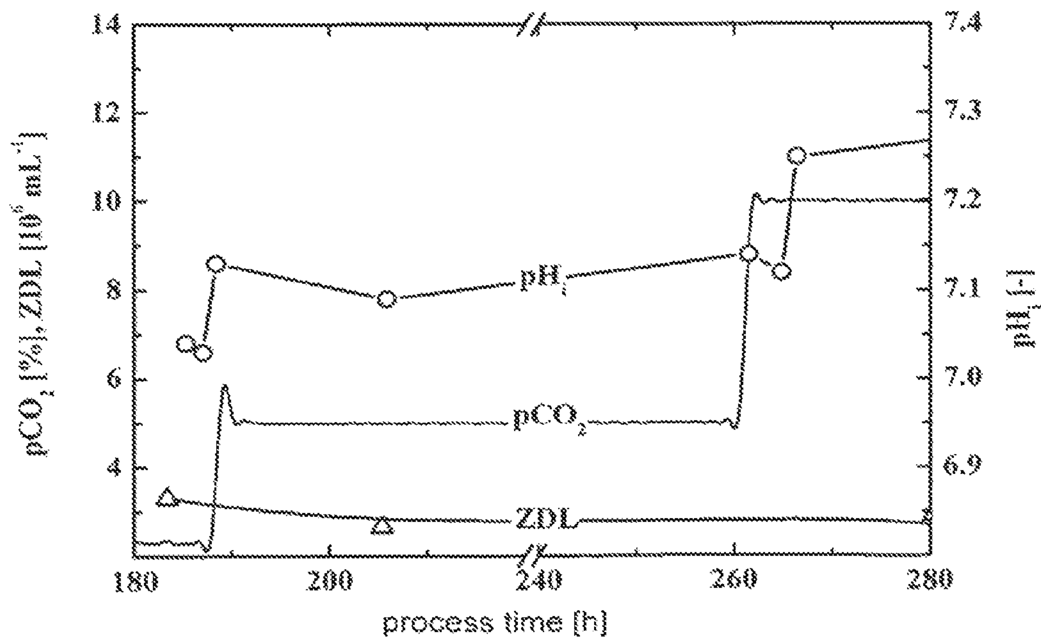

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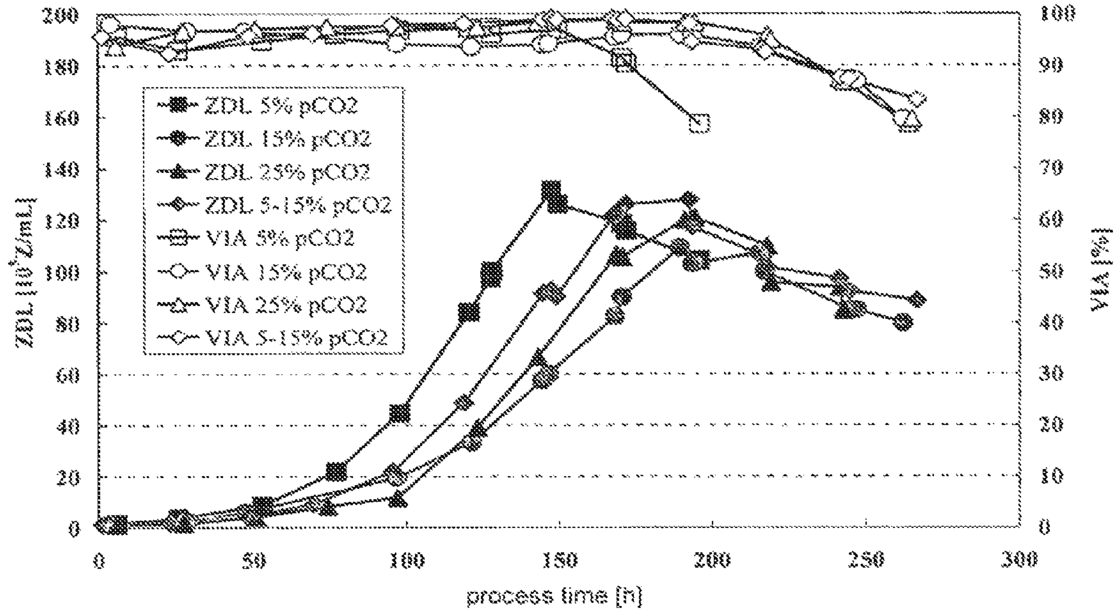

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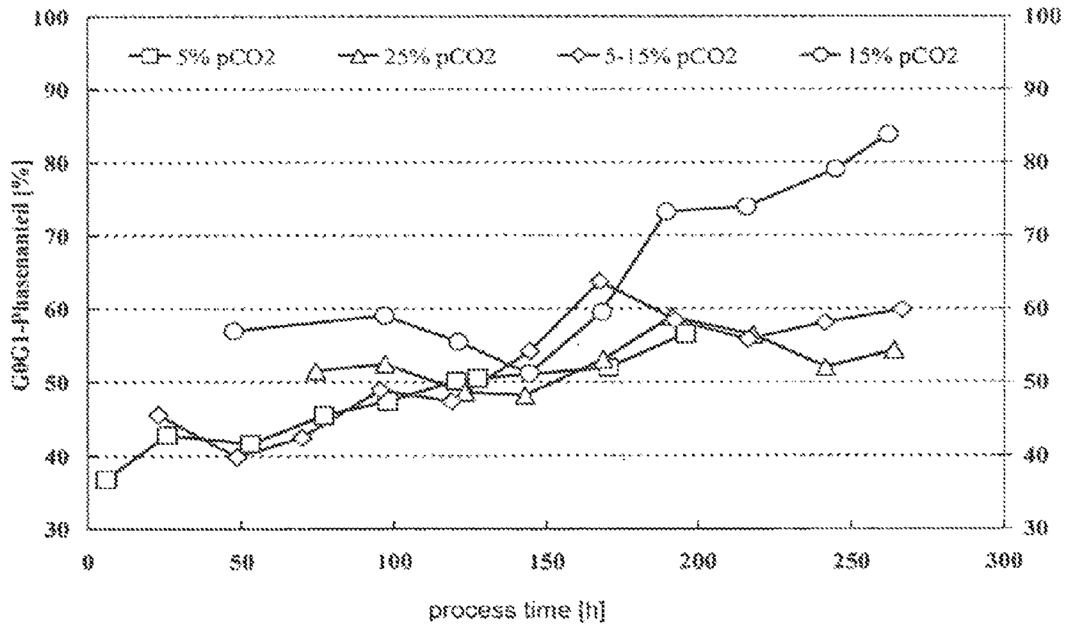

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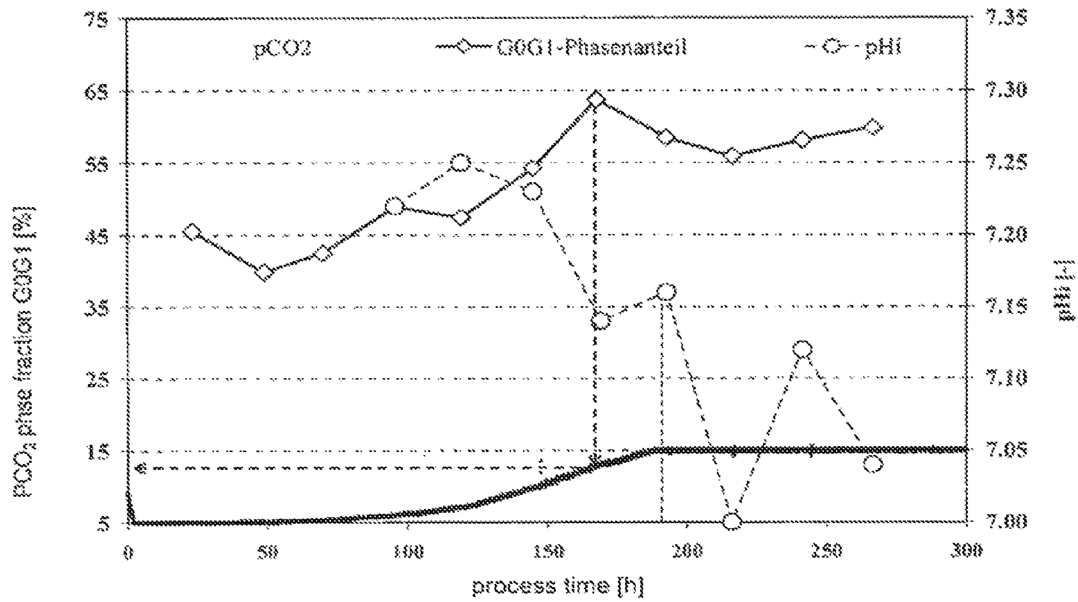

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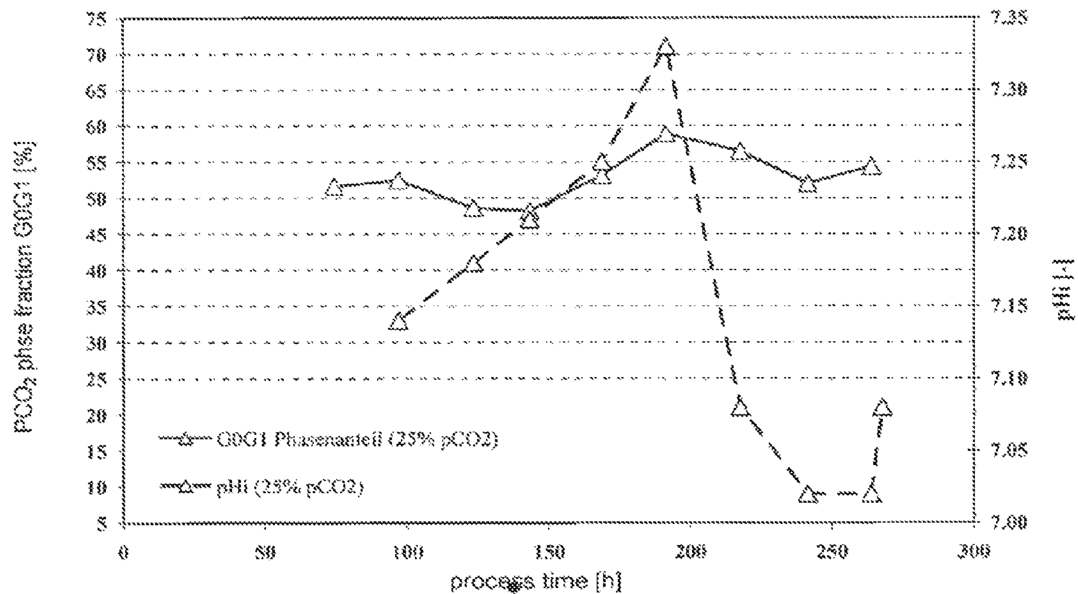

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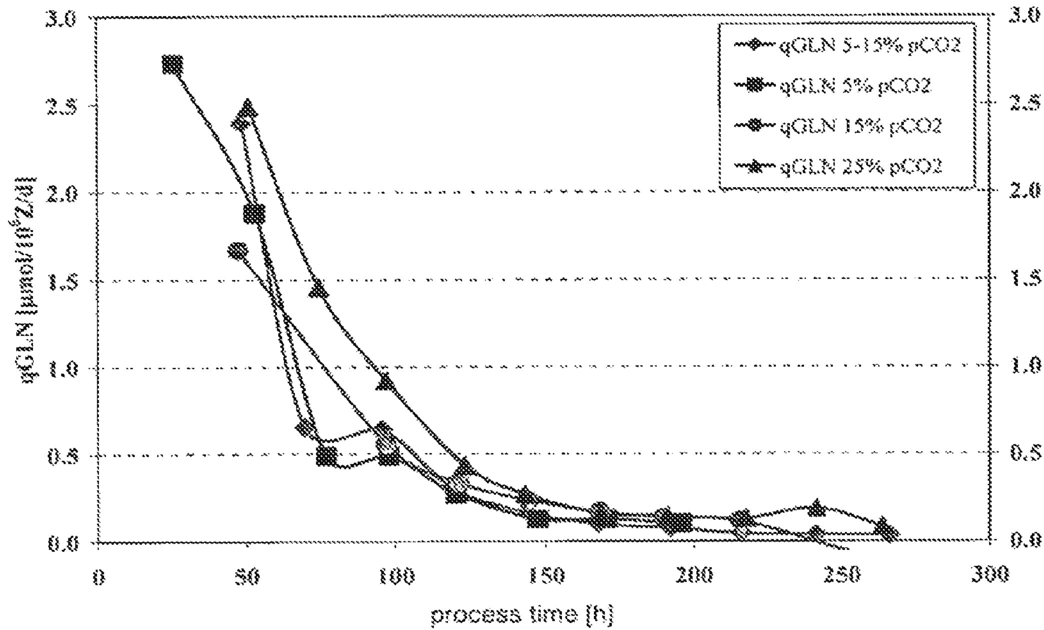

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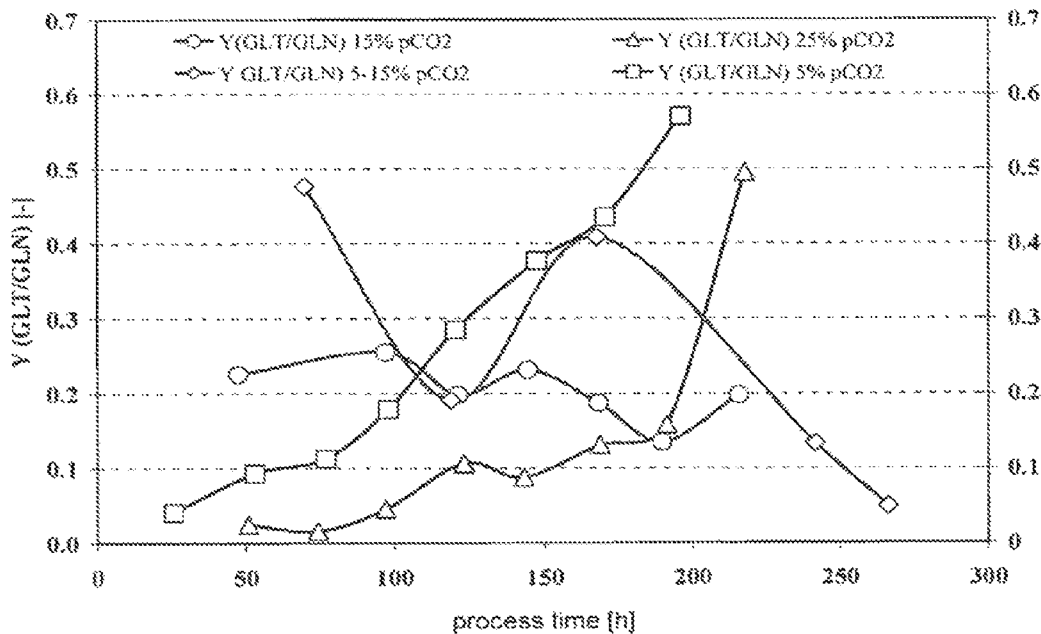

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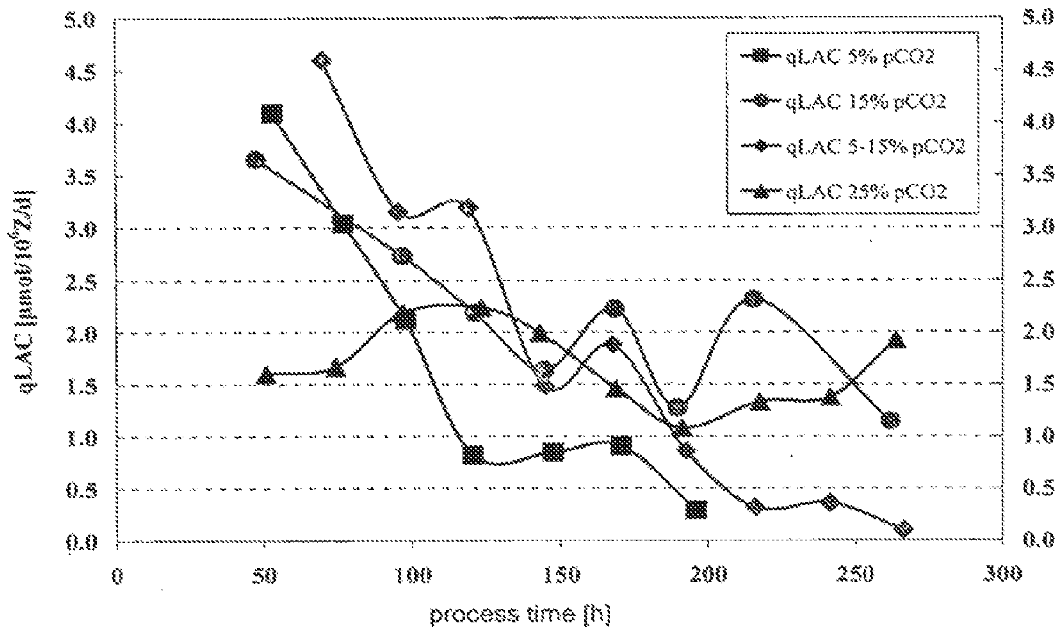

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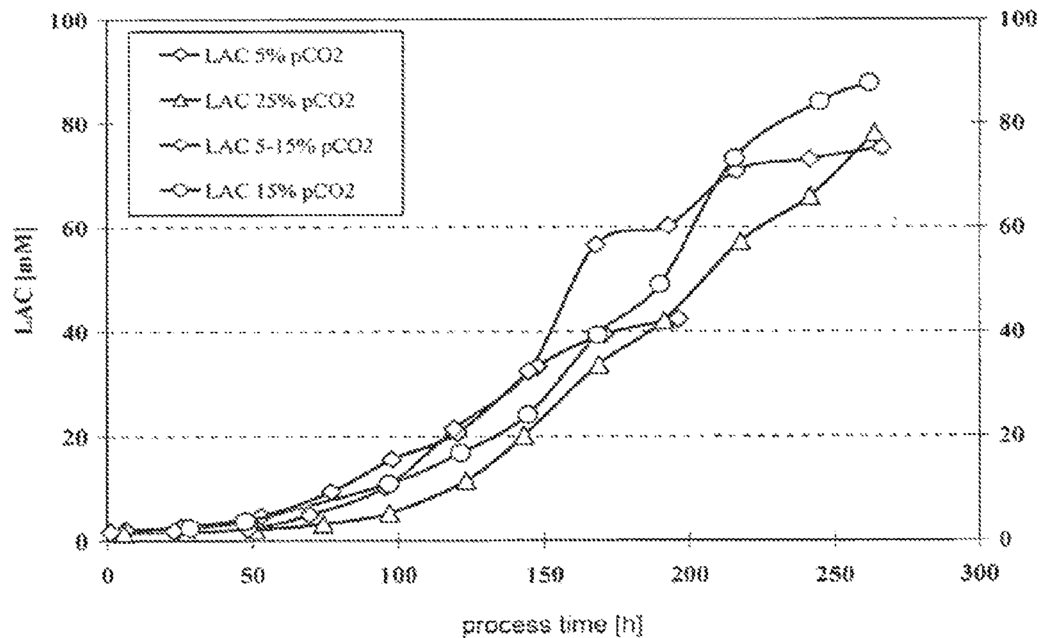

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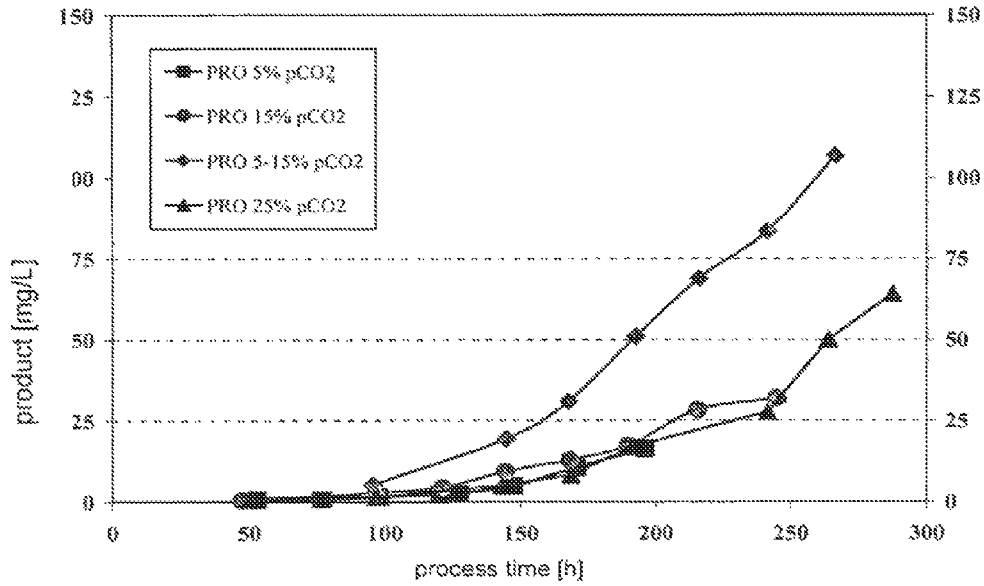

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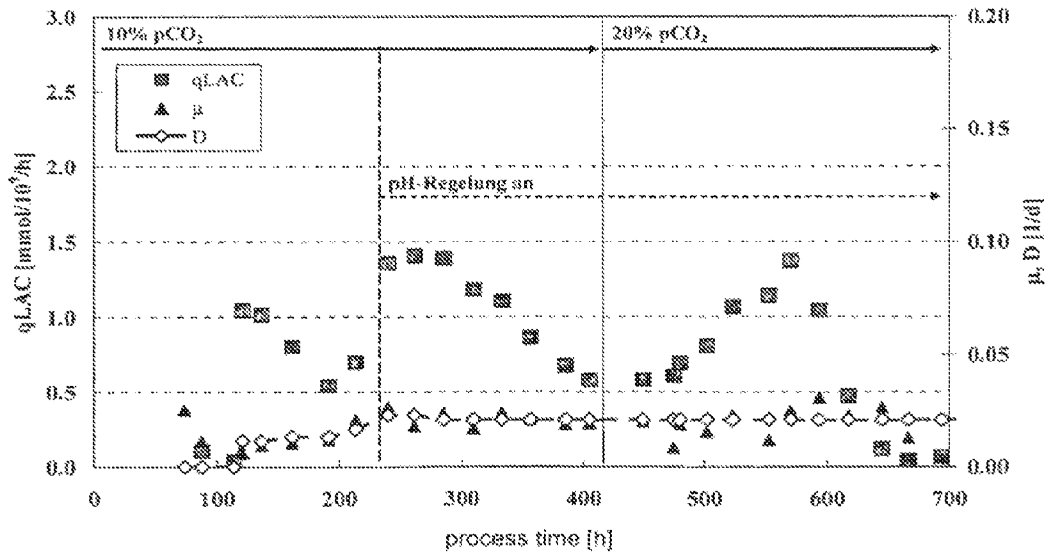

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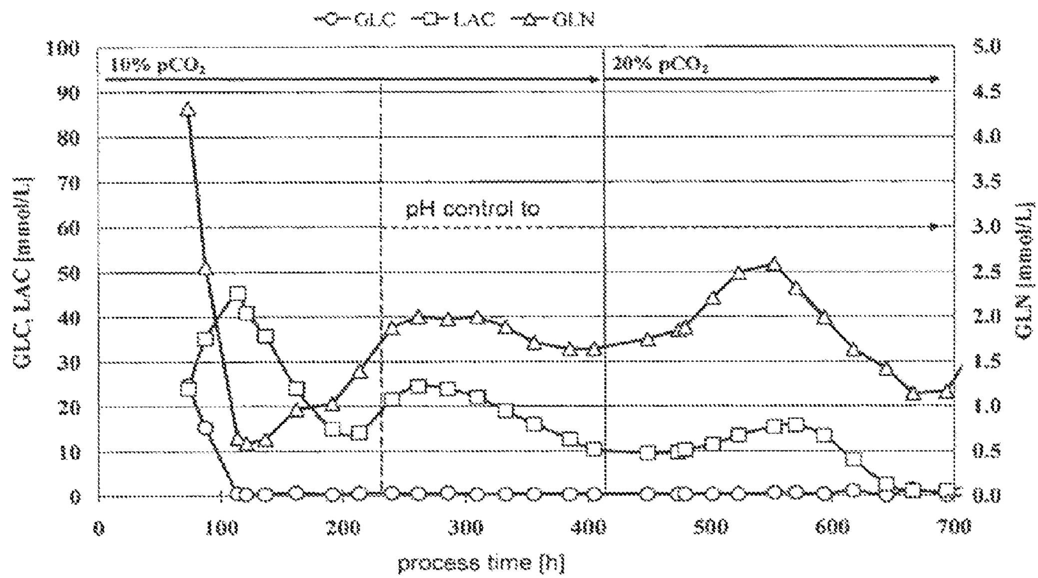

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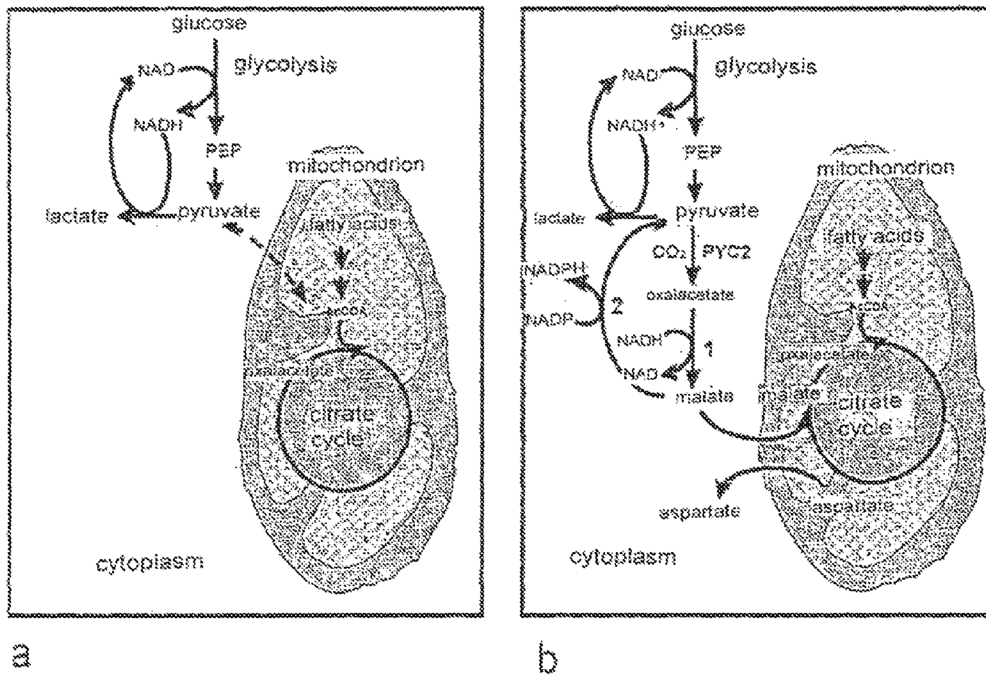

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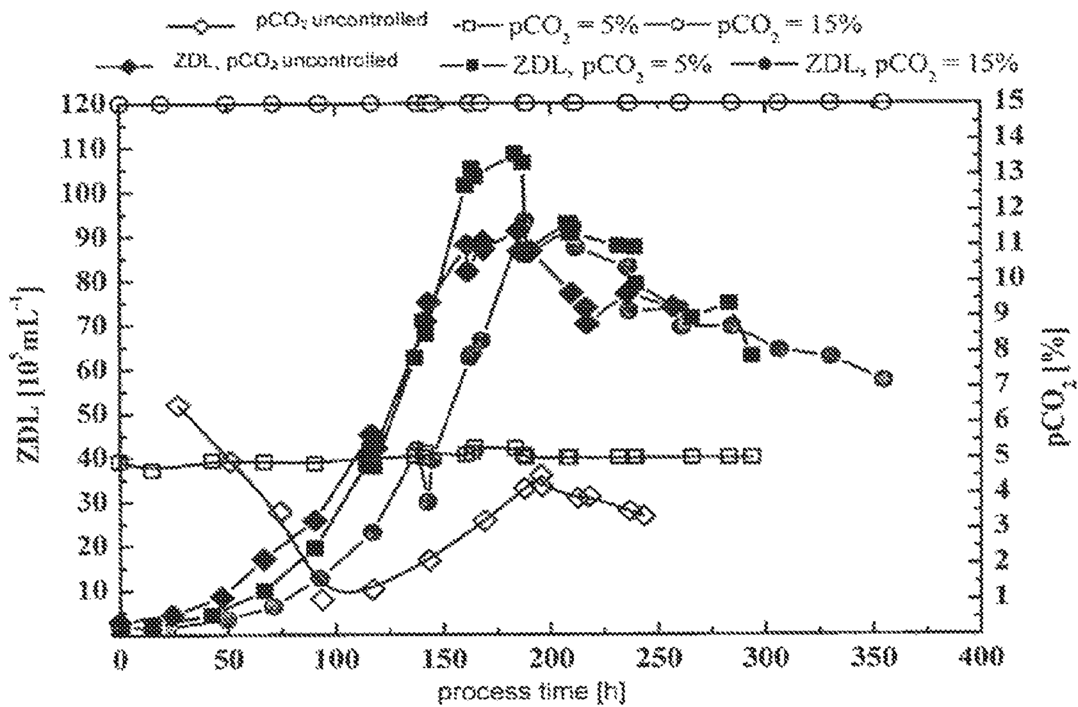

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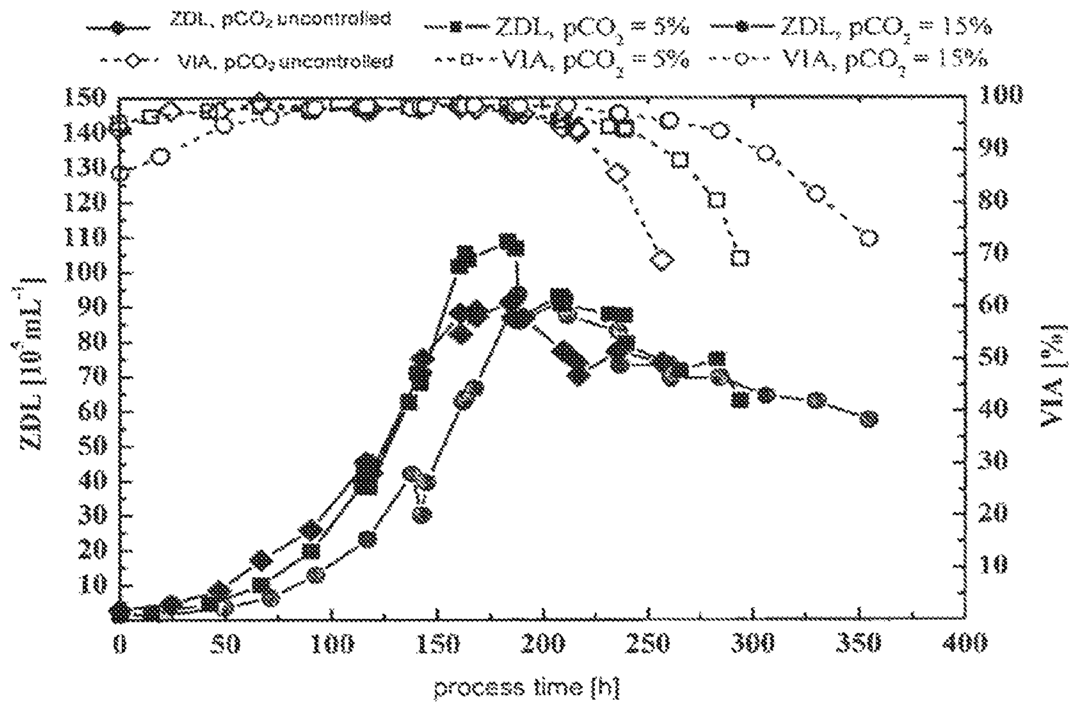

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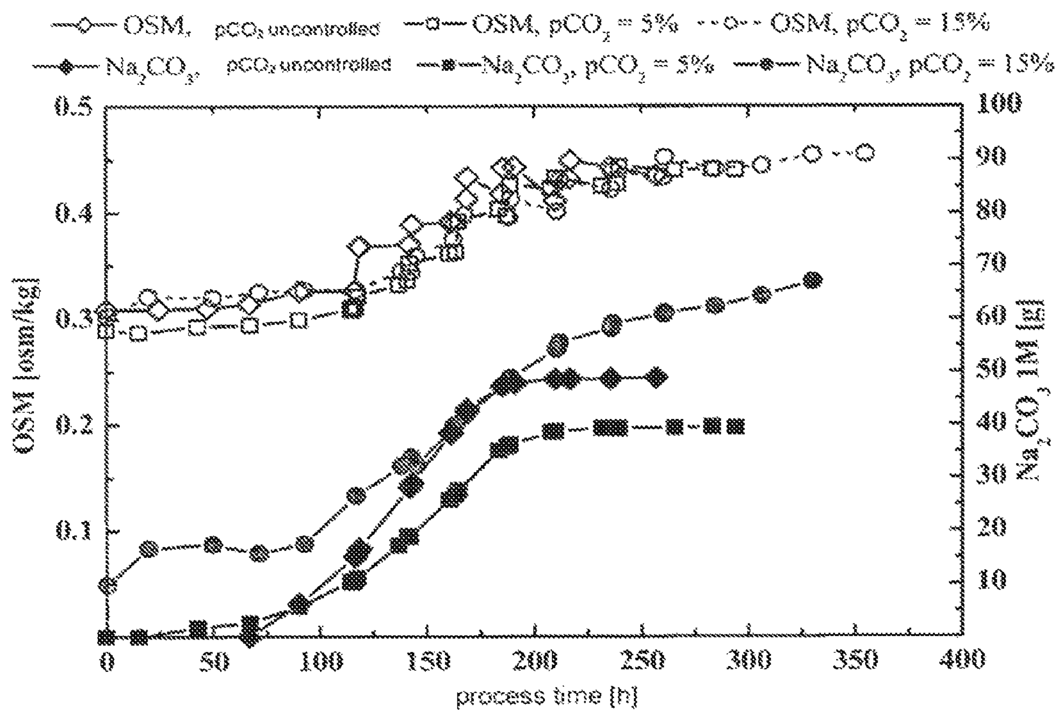

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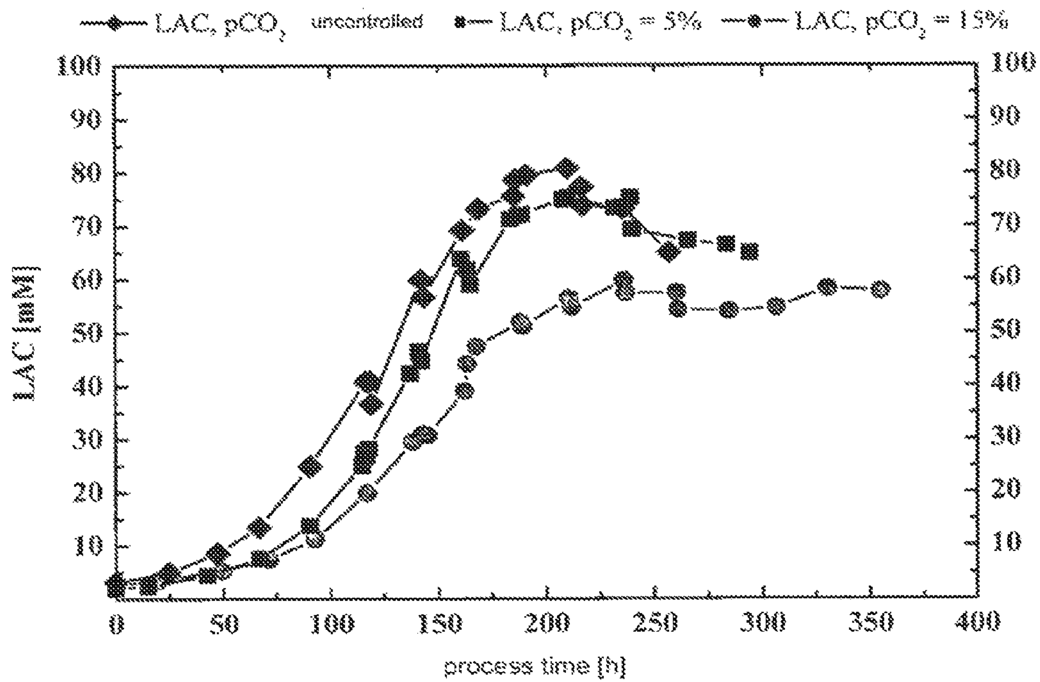

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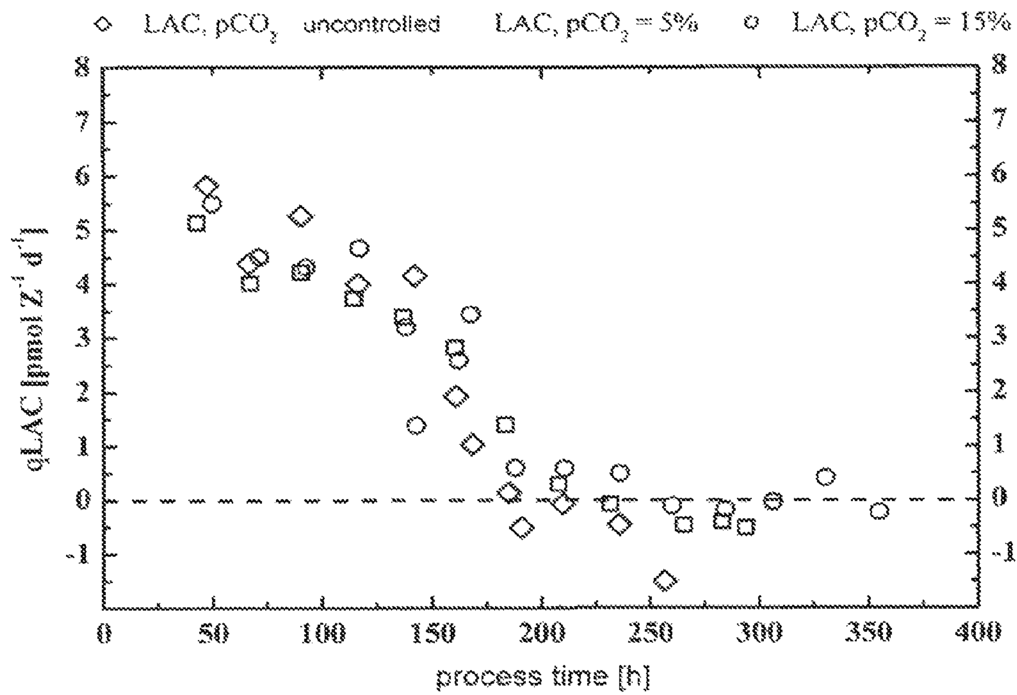

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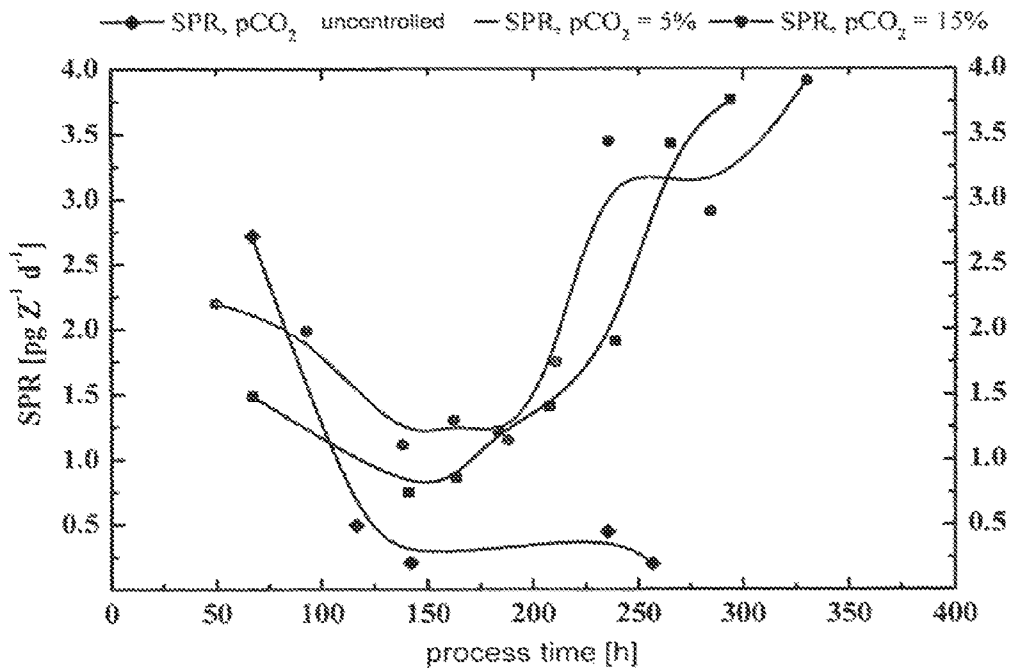

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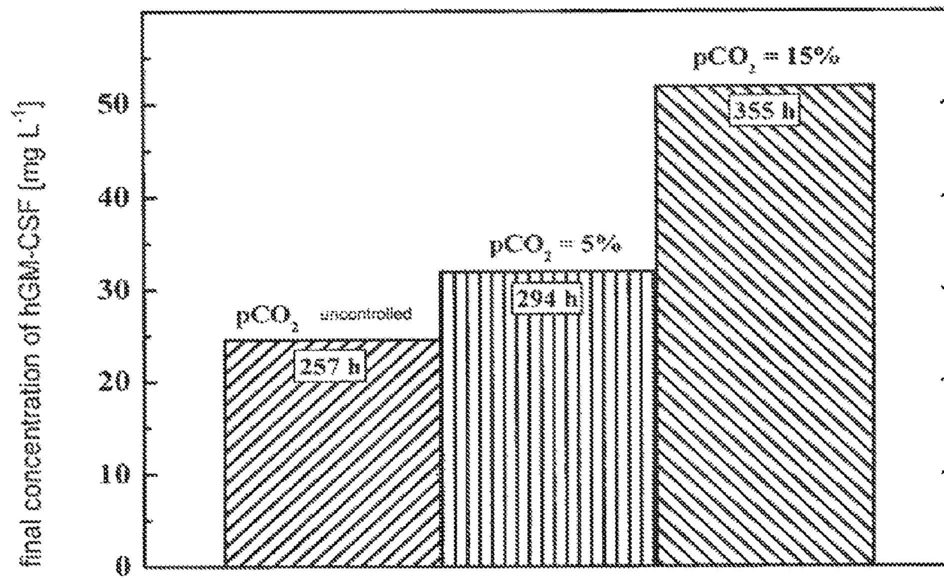

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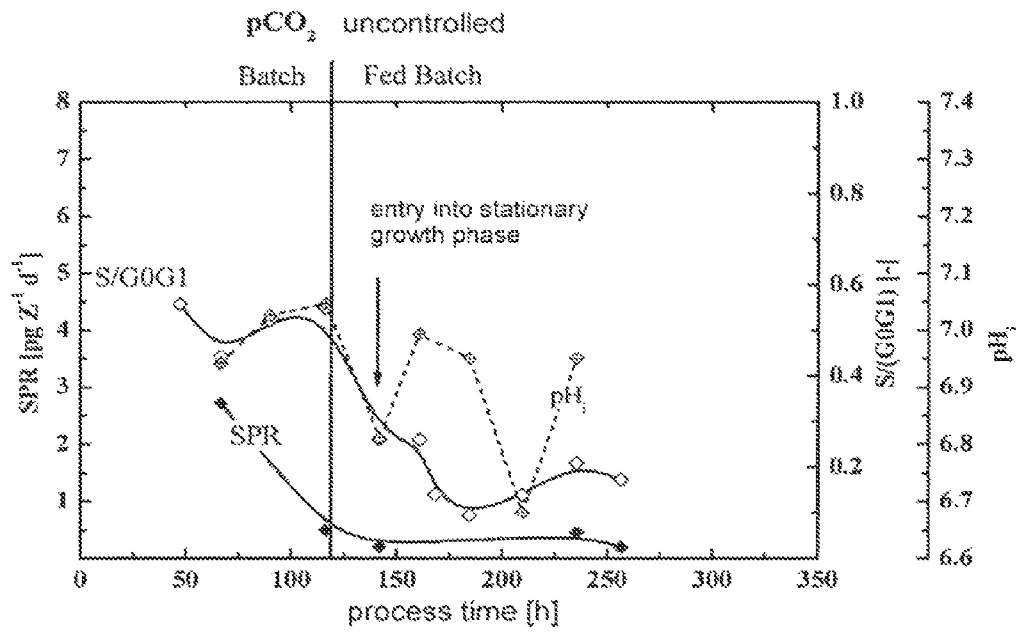

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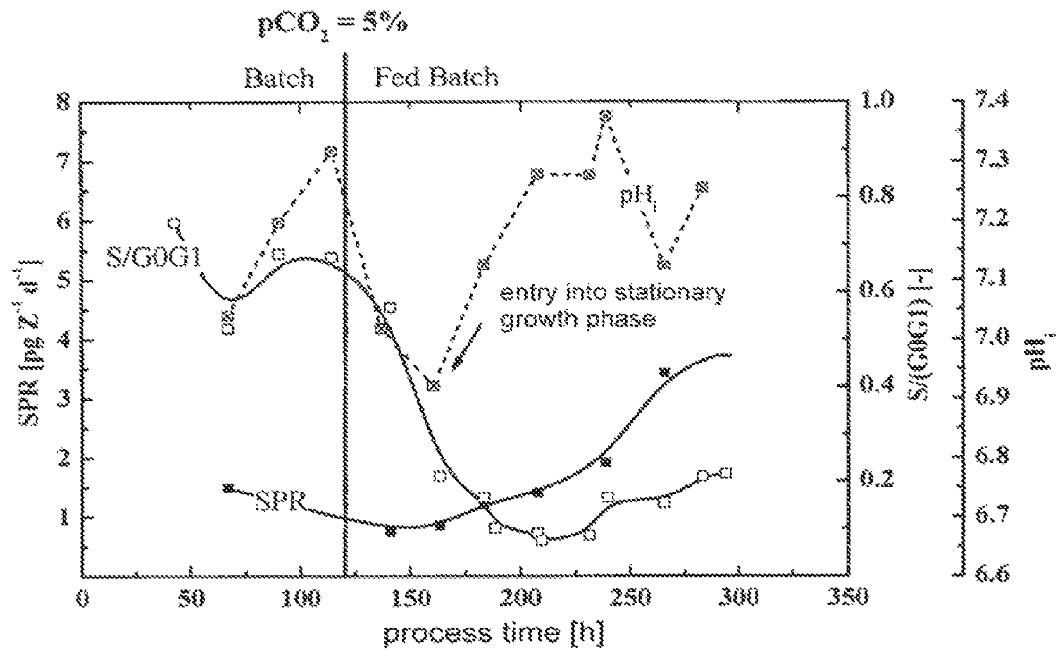

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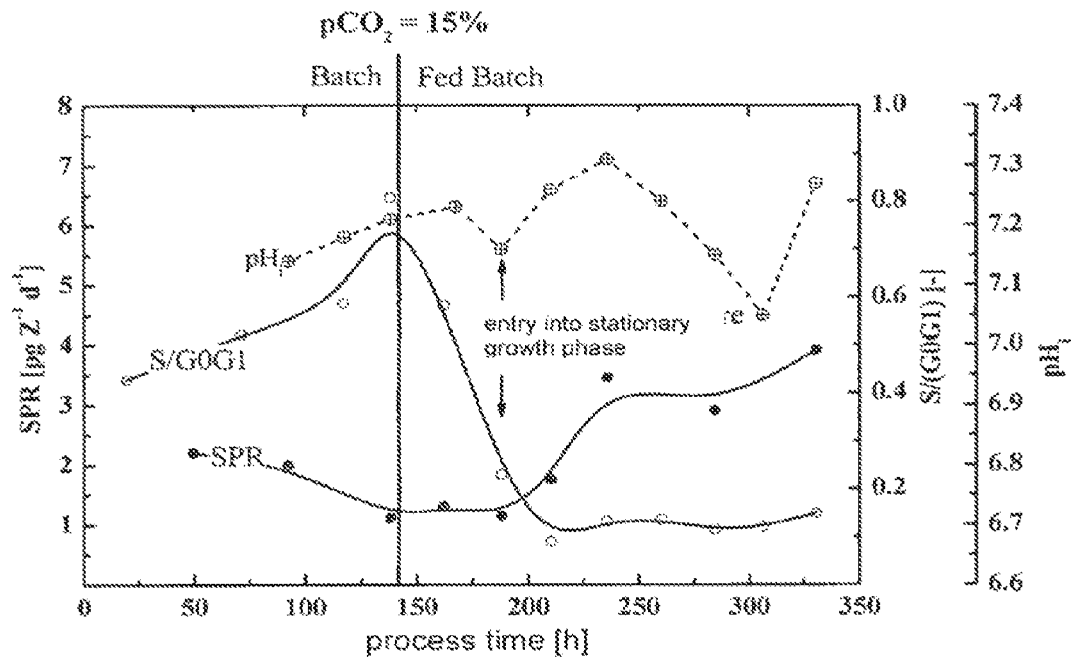

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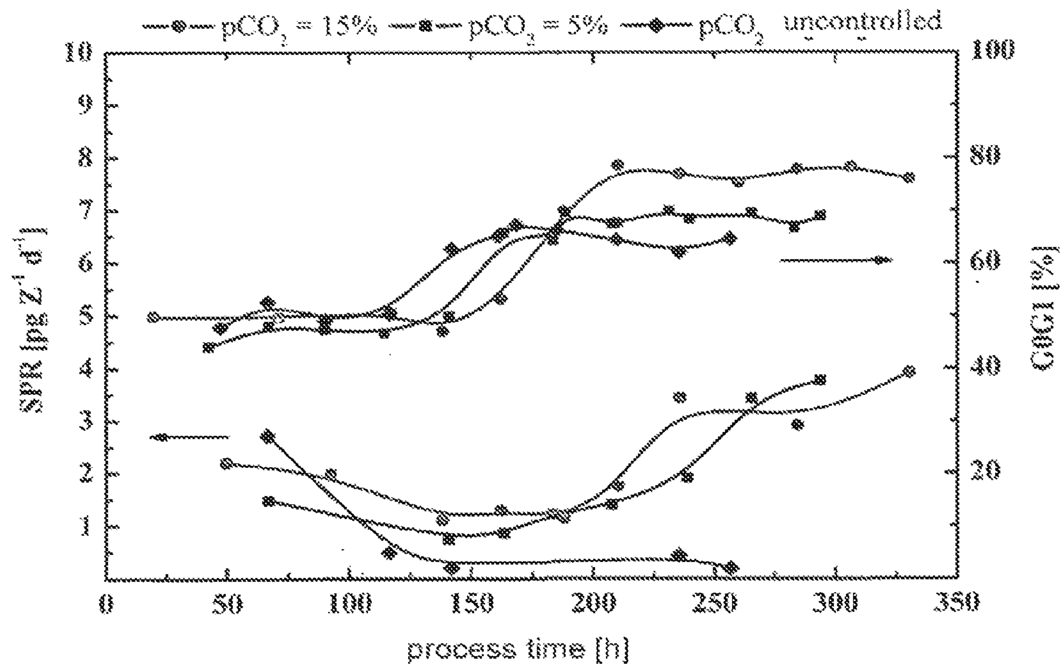

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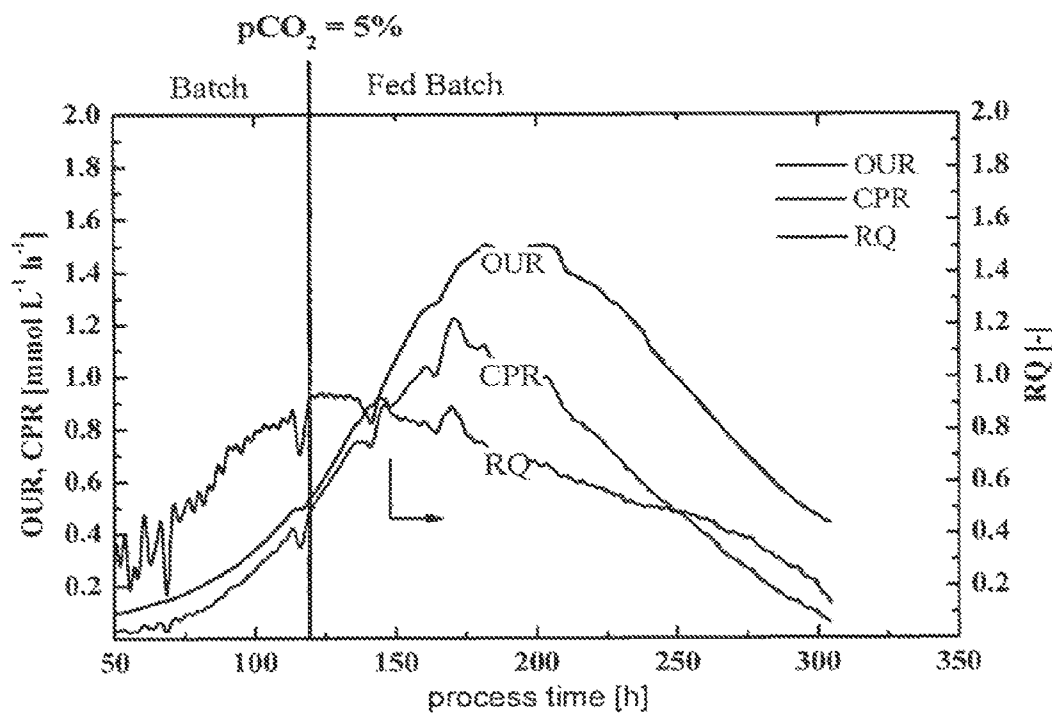

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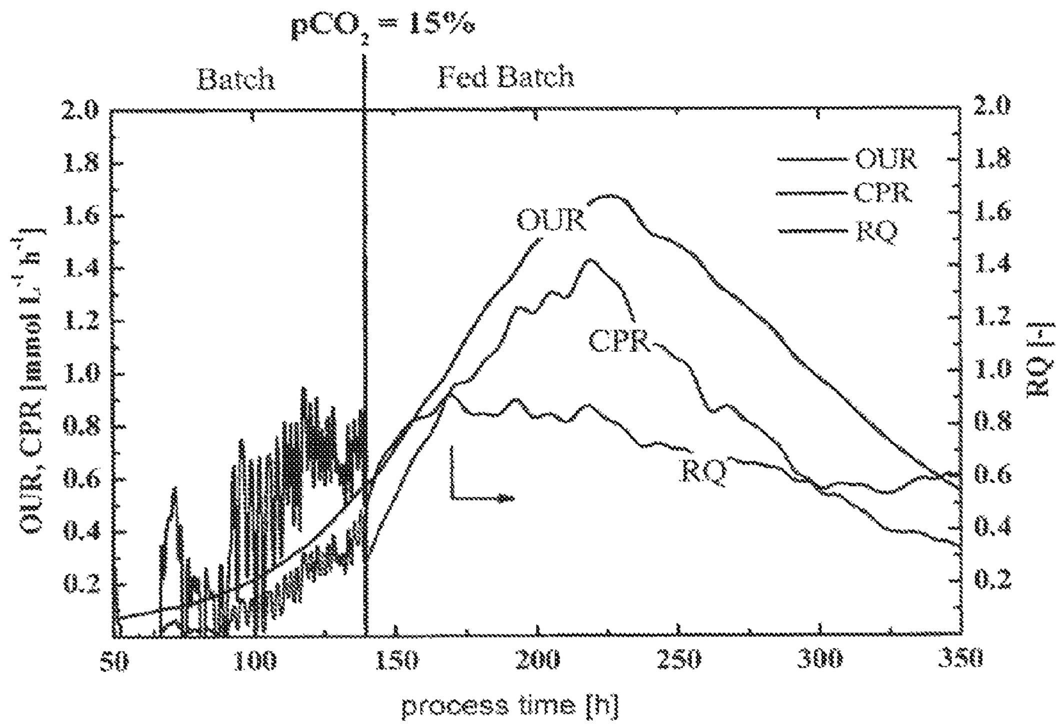


Figure 50

EXHIBIT BB

US008512983B2

(12) **United States Patent**
Gawlitzek et al.

(10) **Patent No.: US 8,512,983 B2**
(45) **Date of Patent: Aug. 20, 2013**

(54) **PRODUCTION OF PROTEINS IN GLUTAMINE-FREE CELL CULTURE MEDIA**

(76) Inventors: **Martin Gawlitzek**, Redwood City, CA (US); **Shun Luo**, Irvine, CA (US); **Christina Teresa Petraglia**, San Ramon, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 151 days.

(21) Appl. No.: **12/852,377**

(22) Filed: **Aug. 6, 2010**

(65) **Prior Publication Data**

US 2011/0091936 A1 Apr. 21, 2011

Related U.S. Application Data

(60) Provisional application No. 61/232,889, filed on Aug. 11, 2009.

(51) **Int. Cl.**
C12P 21/06 (2006.01)

(52) **U.S. Cl.**
USPC **435/69.1**

(58) **Field of Classification Search**
USPC 435/69.1
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,049,494 A * 9/1977 Tomei 435/239
4,399,216 A 8/1983 Axel et al.
4,515,893 A 5/1985 Kung et al.
4,560,655 A 12/1985 Baker
4,657,866 A 4/1987 Kumar
4,766,075 A 8/1988 Goeddel et al.
4,767,704 A 8/1988 Cleveland et al.
4,816,567 A 3/1989 Cabilly et al.
4,853,330 A 8/1989 Goeddel et al.
4,927,762 A 5/1990 Darfler
5,091,313 A 2/1992 Chang
5,122,469 A 6/1992 Mather et al.
5,185,259 A 2/1993 Goeddel et al.
5,500,362 A 3/1996 Robinson et al.
5,545,806 A 8/1996 Lonberg et al.
5,545,807 A 8/1996 Surani et al.
5,569,825 A 10/1996 Lonberg et al.
5,622,700 A 4/1997 Jardieu et al.
5,625,126 A 4/1997 Lonberg et al.
5,633,425 A 5/1997 Lonberg et al.
5,641,870 A 6/1997 Rinderknecht et al.
5,661,016 A 8/1997 Lonberg et al.
5,672,347 A 9/1997 Aggarwal et al.
5,693,762 A 12/1997 Queen et al.
5,714,338 A 2/1998 Wai Fei et al.
5,721,108 A 2/1998 Robinson et al.
5,725,856 A 3/1998 Hudziak et al.
5,736,137 A 4/1998 Anderson et al.
5,750,373 A 5/1998 Garrard et al.
5,776,456 A 7/1998 Anderson et al.
5,821,333 A 10/1998 Carter et al.
5,821,337 A 10/1998 Carter et al.
5,843,439 A 12/1998 Anderson et al.
6,075,181 A 6/2000 Kucherlapati et al.
6,150,584 A 11/2000 Kucherlapati et al.
6,171,586 B1 1/2001 Lam et al.
6,194,551 B1 2/2001 Idusogie et al.
6,242,195 B1 6/2001 Idusogie et al.
6,399,061 B1 6/2002 Anderson et al.
6,455,043 B1 9/2002 Grillo-Lopez
6,528,624 B1 3/2003 Idusogie et al.
6,538,124 B1 3/2003 Idusogie et al.
6,610,516 B1 8/2003 Anderson et al.
6,982,321 B2 1/2006 Winter
7,060,269 B1 6/2006 Baca et al.
7,087,409 B2 8/2006 Barbas, III et al.
7,169,901 B2 1/2007 Baca et al.
7,297,334 B2 11/2007 Baca et al.
2002/0004587 A1 1/2002 Miller et al.
2002/0006404 A1 1/2002 Hanna
2002/0009444 A1 1/2002 Grillo-Lopez
2002/0012665 A1 1/2002 Hanna
2002/0058029 A1 5/2002 Hanna
2002/0128488 A1 9/2002 Yamakawa et al.
2002/0197255 A1 12/2002 Anderson et al.
2002/0197256 A1 12/2002 Grewal
2003/0021781 A1 1/2003 Anderson et al.
2003/0082172 A1 5/2003 Anderson et al.
2003/0095963 A1 5/2003 Anderson et al.
2003/0103971 A1 6/2003 Hariharan et al.
2003/0147885 A1 8/2003 Anderson et al.

(Continued)

FOREIGN PATENT DOCUMENTS

EP 0117058 8/1990
EP 0117059 12/1991

(Continued)

OTHER PUBLICATIONS

Nagle et al. 1971; An improved heat-stable glutamine-free chemically defined medium for growth of mammalian cells. J. Cell Physiol. 77: 259-264.*

(Continued)

Primary Examiner — Karen Cochrane Carlson
(74) *Attorney, Agent, or Firm* — Connie Wong; Christopher De Vry; Arnold & Porter LLP

(57) **ABSTRACT**

The present invention relates generally to glutamine-free cell culture media supplemented with asparagine. The invention further concerns the production of recombinant proteins, such as antibodies, in asparagine-supplemented glutamine-free mammalian cell culture.

25 Claims, 25 Drawing Sheets

(56) **References Cited**

U.S. PATENT DOCUMENTS

2004/0186051	A1	9/2004	Kelley et al.	
2005/0070689	A1 *	3/2005	Dixit et al.	530/350
2006/0246004	A1	11/2006	Adams et al.	

FOREIGN PATENT DOCUMENTS

EP	0307247	8/1995
EP	0404097	8/1997
EP	0117060	8/2007
EP	0420937	9/2008
EP	2464725	11/2012
WO	WO 87/00195	1/1987
WO	WO 89/05859	6/1989
WO	WO 90/03430	4/1990
WO	WO 91/00358	1/1991
WO	WO 91/10741	7/1991
WO	WO 93/04173	3/1993
WO	WO 93/11161	6/1993
WO	WO 95/19181	7/1995
WO	WO 95/23865	9/1995
WO	WO 96/30046	10/1996
WO	WO 96/33735	10/1996
WO	WO 96/34096	10/1996
WO	WO 96/40210	12/1996
WO	WO 97/25428	7/1997
WO	WO 97/26912	7/1997
WO	WO 98/06248	2/1998
WO	WO 98/23761	6/1998
WO	WO 98/24893	6/1998
WO	WO 98/45331	10/1998
WO	WO 98/51793	11/1998
WO	WO 98/56418	12/1998
WO	WO 98/58964	12/1998
WO	WO 99/22764	5/1999
WO	WO 99/51642	10/1999
WO	WO 00/09160	2/2000
WO	WO 00/27428	5/2000
WO	WO 00/27433	5/2000
WO	WO 00/42072	7/2000
WO	WO 00/44788	8/2000
WO	WO 00/67796	11/2000
WO	WO 00/75348	12/2000
WO	WO 01/03734	1/2001
WO	WO 01/10460	2/2001
WO	WO 01/10461	2/2001
WO	WO 01/10462	2/2001
WO	WO 01/40309	6/2001
WO	WO 01/74388	10/2001
WO	WO 01/77342	10/2001
WO	WO 01/80884	11/2001
WO	WO 01/97858	12/2001
WO	WO 02/04021	1/2002
WO	WO 02/34790	5/2002
WO	WO 02/060955	8/2002
WO	WO 02/079255	10/2002
WO	WO 02/096948	12/2002
WO	WO 2004/092219	10/2004
WO	2006083971	* 10/2006

OTHER PUBLICATIONS

Kurano et al. 1990; Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor. 2. Effects of medium components and waste products. J. Biotechnol. 15: 113-128.*

www. products.invitrogen.com/ivgn/product/A1049001; 2012.*

Sanfeliu et al. 1999; Effect of glutamine limitation on the death of attached Chinese hamster ovary cells. Biotechnol. Bioeng. 64: 46-53.*

www. sigmaaldrich.com/life-science/cell-culture/learning-center/media-formulations; 2012.*

De la Cruz Edmonds et al. 2006; Development of transfection and high-producer screening protocols for the CHOK1SV cell system. Molecular Biotechnology 34: 179-190.*

www. Invitrogen.com/1/1/10163-cd-cho-medium-1x-liquid.html; 2012.*

Wu et al. 2001; Multimerization of chimeric anti-CD20 single-chain Fv-Fc fusion protein is mediated through variable domain exchange. Protein Engineering 14(12): 1025-1033.*

Beck et al. 2008; Trends in glycosylation, glycoanalysis, and glycoengineering of therapeutic antibodies and Fc-Fusion proteins. Current Pharmaceutical Biotechnology 9(6): 482-501.*

Haiyan Huang, et al., "Nitrogen metabolism of asparagine and glutamate in Vero cells studied by 1H/15N NMR spectroscopy", Applied Microbiology and Biotechnology, Springer, Berlin, DE LNKD-DOI: 10.1007/S00253-007-1181-8, vol. 77, No. 2, Oct. 19, 2007, pp. 427-436, XP019560711, ISNN: 1432-0614.

Kurano N., et al., "Growth behavior of Chinese hamster ovary cells in a compact loop biorcactor. 2. Effects of medium components and waste products", Journal of Biotechnology, Elsevier Science Publishers, Amsterdam, NL LNKD-DOI: 10.1016/0168-1656 (90) 90055-G, vol. 15, No. 1-2, Jul. 1, 1990, pp. 113-128, XP023944043, ISNN: 0168-1656, [retrieved on Jul. 1, 1990].

GB 2 237 288 A (Celltech Ltd [GB]), May 1, 1991, Abstract, p. 4, paragraphs 2, 3, 5; claim 8.

Altamirano, et al., "Strategies for fed-batch cultivation of t-PA producing CIIO cells: substitution of glucose and glutamine and rational design of culture medium", Journal of Biotechnology, 110: 171-179, (2004).

Miller, et al., "A kinetic analysis of hybridoma growth and metabolism in batch and continuous suspension culture: Effect of nutrient concentration, dilution rate, and pH", Biotechnology and Bioengineering, vol. 32, pp. 947-965, (1988).

Sanfeliu, et al., "Effect of Glutamine limitation on the death of attached Chinese hamster ovary cells", Biotechnology and Bioengineering, 64: pp. 46-53, (1999).

Schneider, et al., "The importance of ammonia in mammalian cell culture", Journal of Biotechnology, 46: 161-185, (1996).

Aruffo et al. "CD44 is the Principal Cell Surface Receptor for Hyaluronate", Cell vol. 61, 1303-1313, 1990.

Barbas et al. "In Vitro Evolution of a Neutralizing Human Antibody to Human Immunodeficiency Virus Type 1 to Enhance Affinity and Broaden Strain Cross-Reactivity", Proc. Natl. Acad. Sci. USA; vol. 91, pp. 3809-3813, 1994.

Barnes and Sato "Methods for Growth of Cultured Cells in Serum-Free Medium", Analytical Biochemistry 102: 255, 1980.

Barnes et al. "Serum-Free Cell Culture: a Unifying Approach", Cell, vol. 22, 649-655,1980.

Baumann et al. "Dexammethasone Regulates the Program of Secretory Glycoprotein Synthesis in Hepatoma Tissue Culture Cells", J. Cell Biology, vol. 85, pp. 1-8, 1980.

Bird et al. "Single-Chain Antigen-Binding proteins", Science vol. 242, pp. 423-426, 1988.

Boerner et al "Production of antigen-specific human monoclonal antibodies from in vitro-primed human splenocytes:", J. of Immunology, vol. 147, No. 1, pp. 86-95, 1991.

Brennan et al. "Preparation of Bispecific Antibodies by Chemical Recombination of Monoclonal Immunoglobulin G_1 Fragments", Science 229:81, 1985.

Brodeur et al., Monoclonal Antibody Production Techniques and Applications, pp. 51-63, 1987 (Marcel Dekker, Inc., New York).

Bruggemann et al. "Designer Mice: The Production of Human Antibody Repertoires in Transgenic Animals", Year Immunol. vol. 7, p. 33-40, 1993.

Burton, D.R. "Immunoglobulin G:Functional Sites", Molecular Immunology vol. 22:161-206, 1985.

Capel et al. "Heterogeneity of human IgG Fc receptors", Immunomethods vol. 4, pp. 25-34, 1994.

Carter et al. "High Level *Escherichia coli* Expression and Production of a Bivalent Humanized Antibody Fragment", Biotechnology 10:163-167, 1992.

Clackson et al. "Making antibody fragments using phage display libraries", Nature, vol. 352, pp. 624-628, 1991.

Clynes et al. "Fc receptors are required in passive and active immunity to melanoma", Proc. Natl. Acad. Sci. (USA); vol. 95, pp. 652-656, 1998.

Cole et al. "The EBV-Hybridoma Technique and its application to Human Lung Cancer" Monoclonal Antibodies and Cancer Therapy, pp. 77-96, 1985.

Daeron M. "Fc Receptor Biology", Annu. Rev. Immunol.: 15: 203-34, 1997.
Duchosal et al. "Immunization of hu-PBL-SCID Mice and the Rescue of Human Monoclonal Fab Fragments through Combinatorial Libraries" Nature 355:258, 1992.
Fellouse et al. "Synthetic Antibodies from a Four-Amino-Acid Code: A Dominant role for Tyrosine in Antigen Recognition", PNAS: vol. 101; No. 34: 12467-12472, 2004.
Fishwild et al "High-Avidity Human IgGk Monoclonal Antibodies from a Novel Strain of Minilocus Transgenic Mice", Nature Biotech; vol. 14, pp. 845-851, 1996.
Gasser et al. "Expression of Abbreviated Mouse Dihydrofolate Reductase Genes in Cultured Hamster Cells", Proc. Natl. Acad. Sci. USA vol. 79, pp. 6522-6526, 1982.
Gazzano-Santoro et al. "A Non-Radioactive Complement-Dependent Cytotoxicity Assay for Anti-CD20 Monoclonal Antibody", J of Immunol Methods 202, pp. 163-171, 1997.
Gething et al. "Cell-surface Expression of Influenza Haemagglutinin from a Cloned DNA the RNA Gene", Nature vol. 293: 620-625, 1981.
Ghetie et al. "FcRn: the MHC Class I-Related Receptor that is more than an IgG Transporter" vol. 18, No. 12, pp. 592-598, 1997.
Ghetie et al. "Increasing the Serum Persistence of an IgG Fragment by Random Mutagenesis", Nature Biotech vol. 15, pp. 637-640, 1997.
Goding J.W. "Monoclonal Antibodies: Principles and Practice" pp. 59-103. (Academic Press, 1986).
Graham et al. "Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5", J. Gen. Virol:36, 59-72, 1977.
Graham et al. "A New Technique for the Assay of Infectivity of Human Adenovirus 5 DNA" Virology 52, 456-467, 1973.
Gruber et al. "Efficient Tumor Cell Lysis Mediated by a Bispecific Single Chain Antibody Expressed in *Escherichia coli* " J. Immunol. 152:5368, 1994.
Gueffroy, D. "A Guide for the Preparation and Use of buffers in Biological Systems" Cal Biochem Corporation, 1975.
Guyer et al. "Immunoglobulin Binding by Mouse Intestinal Epithelial Cell Receptors" Journal of Immunology; vol. 117, No. 2, pp. 587-593, 1976.
Haas et al. "FcYreceptors of phagocytes", J. Lab. Clin Med 126: 330-41, 1995.
Ham et al. "Media and Growth Requirements" Meth. Enz., 58: 44, 1979.
Hamers-Casterman et al. "Naturally Occurring Antibodies Devoid of Light Chains", Nature vol. 363, pp. 446-448, 1993.
Hammerling et al. "Monoclonal Antibodies and T-Cell Hybridomas", Elsevier; pp. 563-681, 1981.
Harris et al. "Production of Humanized Monoclonal Antibodies for in Vivo Imaging and Therapy", Biochemical Society Transactions; vol. 23, pp. 1035-1038, 1995.
Hawkins et al. "Selection of Phage Antibodies by Binding Affinity Mimicking Affinity Maturation", J. Mol. Biol.; vol. 226, pp. 889-896, 1992.
Hinton et al. "Engineered Human IgG Antibodies with Longer Serum Half-lives in Primates", J. Biol. Chemistry, vol. 279, No. 8, pp. 6213-6216, 2004.
Holliger et al. "Diabodies: Small Bivalent and Bispecific Antibody Fragments" Proc. Natl. Acad. Sci. USA; vol. 90, pp. 6444-6448, 1993.
Hongo et al. Development and Characterization of Murine Monoclonal Antibodies to the Latency-Associated Peptide of Transforming Growth Factor β1 ; Hybridoma; vol. 14, No. 3, pp. 253-260, 1995.
Hoogenboom et al. "By-passing Immunisation Human Antibodies from Synthetic Repertoires of Germline Vh Gene Segments Rearranged in Vitro", J. Mol. Biol. vol. 227, pp. 381-388, 1992.
Hoogenboom et al. "Construction and Expression of Antibody-Tumor Necrosis Factor Fusion Proteins", Mol. Immunol. vol. 28, No. 9, pp. 1027-1037, 1991.
Hudson et al. "Engineered Antibodies" Nature Med. vol. 9, pp. 129-134, 2003.
Hurle et al. "Protein Engineering Techniques for Antibody Humanization", Current Opinion Biotech.: 5: 428-433, 1994.
Huston et al. "Protein Engineering of Antibody Binding Sites: Recovery of Specific Activity in an Anti-Digoxin Single-Chain Fv Analogue Produced in *Escherichial coli* ", PNAS (USA); vol. 85, pp. 5879-5883, 1988.
Idusogie et al. "Mapping of the C1q Binding Site on Rituxan, a Chimeric Antibody with a Human IgG1 Fc", J. Immunol. vol. 164: 4178-4184, 2000.
Jackson et al. "In Vitro Antibody Maturation", J. Immunol: 154:3310-3319, 1995.
Jakobovits et al. "Analysis of Homozygous Mutant Chimeric Mice: Deletion of the Immunoglobulin Heavy-Chain Joining Region Blocks B-cell Development and Antibody Production" Proc. Natl. Acad. Sci. USA; vol. 90, pp. 2551-2555, 1993.
Jakobovits et al. "Gerin-line Transmission and Expression of a Human-derived Yeast Artificial Chromosome", Nature, vol. 362, pp. 255-258, 1993.
Johnson et al. "The Kabat Database and a Bioinformatics Example", Methods in Molecular Biology; vol. 248, pp. 11-25, 2003.
Jones et al. "Replacing the Complementarity-Determining Regions in a Human Antibody with those from a mouse" Nature, vol. 321, pp. 522-525, 1986.
Kaufman et al. "Amplification and Expression of Sequences Cotransfected with a Modular Dihydrofolate Reductase Complementary DNA Gene", J. Mol. Biol. 159: 601-621, 1982.
Keown et al. "Methods for Introducing DNA into Mammalian Cells", Methods in Enzymology: 185:527-537, 1990.
Kohler et al. "Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity", Nature, vol. 256, pp. 495-497, 1975.
Kostelny et al. "Formation of a Bispecific Antibody by the Use of Leucine Zippers" J. Immunol. 148(5):1547-1553, 1992.
Kozbor et al. "A Human Hybrid Myeloma for Production of Human Monoclonal Antibodies", Journal Immunology 133:3001, 1984.
Lee et al. "High-affinity Human Antibodies from Phage-displayed Synthetic Fab Libraries with a Single Framework Scaffold", J. Mol. Biol. 340, 1073-1093, 2004.
Lee et al. "Bivalent Antibody Phage Display Mimics Natural Immunoglobulin", J Immunoligical Methods :284, pp. 119-132, 2004.
Li et al. "Human Antibodies for Immunotherapy Development Generated via a Human B Cell Hybridoma Technology", PNAS (USA) vol. 103, pp. 3557-3562, 2006.
Lonberg et al. "Antigen-Specific Human Antibodies from Mice Comprising Four Distinct Genetic Modifications", Nature: vol. 368, pp. 856-859, 1994.
Lonberg et al. "Human Antibodies from Transgenic Mice" , Intern. Rev. Immunol. vol. 13, pp. 65-93, 1995.
Lubiniecki et al. "Advances in Animal Cell Biology and Technology for Bioprocesses", ESACT pp. 442-451, 1989.
Luckow et al. "Trends in the Development o fBaculovirus Expression Vectors", Biotechnology, vol. 6, pp. 47-55, 1988.
Maeda et al. "Production of Human α-interferon in Silkworm Using a Baculovirus Vector", Nature, vol. 315, pp. 592-594, 1985.
Mantei et al. "Rabbit β-globin Mrna Production in Mouse L Cells Transformed with Cloned Rabbit β-globin Chromosomal DNA", Nature, 281:40-46, 1979.
Mansour et al. "Disruption of the Proto-Oncogene *int*-2 in Mouse Embryo-Derived Stem Cells: a General Strategy for Targeting Mutations to Non-selectable Genes", Nature, 336:348-352, 1988.
Marks et al. "By-passing Immunization Human Antibodies from V-gene Libraries Displayed, on Phage" J. Mol. Biol. vol. 222, pp. 581-597, 1991.
Marks et al. "By-passing Immunization: Building High Affinity Human Antibodies by Chain Shuffling" Biotechnology; vol. 10 pp. 779-783, 1992.
Mather, J.P. "Establishment and Characterization of Two Distinct Mouse Testicular Epithelial Cell Lines" Biology of Reproduction 23, p. 243-252 ,1980.
Mather et al. "Culture of Testicular Cells in Hormone-Supplemented Serum-Free Medium", NYAS ; vol. 0383-0044, p. 44, 1982.
Maxam et al. "Sequencing End-Labeled DNA with Base-Specific Chemical Cleavages", Methods in Enzymology, 65:499, 1980.
McCafferty et al. "Phage Antibodies: Filamentous Phage Displaying Antibody Variable Domains", Nature, 348:552-554, 1990.

Messing et al. "A System for Shotgun DNA Sequencing", Nuceleic Acids Research 9:309, 1981.
Miller et al. Genetic. Engineering Setlow; Penum Publishing; vol. 8, pp. 277-279, 1986.
Millstein et al. "Hybrid Hybridomas and their use in Immunohistochemistry", Nature vol. 305:537-539, 1983.
Morimoto et al. Single-step Purification of F(ab')^2 Journal of Biochemical and Biophysical Methods 24:107-117,1992.
Morrison S.L. "Success in Specification" Nature, vol. 368, pp. 812-813, 1994.
Morrison et al. "Chimeric Human Antibody Molecules: Mouse Antigen-binding Domains with Human Constant Region Domains", Proc. Natl. Acad. Sci.USA, vol. 81, pp. 6851-6855,1984.
Neuberger M. "Generating High-Avidity Human Mabs in Mice", Nature, vol. 14, p. 826, 1996.
Pluckthun, A. "The Pharmacology of Monoclonal Antibodies", Springer-Verlag vol. 113, pp. 269-315, 1994.
Presta, L.G. "Antibody Engineering" Current Opinion in Struct Biol. 2:593-596, 1992.
Presta et al. "Humanization of an Antibody Directed Against IgE", J. Immnol., 151:2623-2632, 1993.
Ravetch et al. "Fc Receptors", Annu. Rev. Immunol. 9: 457-92, 1991.
Riechmann et al. "Reshaping Human Antibodies for Therapy", Nature, vol. 332, pp. 323-329, 1988.
Schier et al. "Identification of Functional and Structural Amino-Acid Residues by Parsimonious Mutagenesis", Gene, 169, pp. 147-155, 1996.
Shalaby et al. "Development of Humanized Bispecific Antibodies Reactive with Cytotoxic Lymphocytes and Tumor Cells Overexpressing the HER2 Protooncogene", J. Exp. Med. 175:217-225,1992.
Shaw et al. "A General Method for the Transfer of Cloned Genes to Plant Cells", Gene 23:315, 1983.
Sheets et al. "Efficient Construction of a large Nonimmune Phage Antibody Library: The Production of High-Affinity Human Single-Chain Antibodies to Protein Antigens", PNAS (USA) vol. 95, pp. 6157-6162, 1998.
Sheriff et al. "Redefining the Minimal Antigen-Binding Frgament", Nature Structural Biology vol. 3, pp. 733-736, 1996.
Shields et al. "High Resolution Mapping of the Binding Site on Human IgG1 for FcYRI, FCYRI, FCYRII, and FCYRIII and FcRn Design of IgG1 Variants with Improved Binding to the FCYR"; J. Biol. Chem. vol. 276, No. 9, pp. 6591-6604, 2001.
Sidhu et al. "Phage-displayed antibody libraries of synthetic heavy chain complementarity determining regions" J. Mol . . . Biol. vol. 338, No. 2, pp. 299-310, 2004.
Simonsen et al. "Isolation and expression of an altered mouse dihydrofolate reductase cDNA" Proc. Natl. Acad. Sci. USA, vol. 80, pp. 249-2499, 1983.
Sims et al. "A Humanized CD18 Antibody Can Block Function without Cell Destruction", Journal Immunol., 151:2296 ,1993.
Stamenkovic et al. "The B Lymphocyte Adhesion Molecule CD22 Interacts with Leukocyte Common Antigen CD45RO on T Cells and α2-6 Sialyltransferase, CD75, on B Cells" Cell vol. 66, 1133-1144, 1991.
Thomas, P. "Hybridization of Denatured RNA and small DNA Fragments Transferred to Nitrocellulose"; Proc. Natl. Acad. Sci. USA: 77:5201-5205, 1980.
Traunecker et al. "Bispecific Single Chain Molecules (Janusins) target Cytotoxic Lymphocytes on HIV Infected Cells" , EMBO Journal vol. 10 No. 12 , pp. 3655-3659, 1991.
Trill et al. "Production of Monoclonal Antibodies in COS and CHO cells", Current Opinion Biotech 6:553-560, 1995.
Tutt et al. "Trispecific F(ab')$_3$ Derivatives that Use Cooperative Signaling via the TCR/CD3 Complex and CD2 to Activate and Redirect Resting Cytotoxic T Cells$_1$" J. Immunol: 147:60-69, 1991.
Urlaub et al. "Isolation of Chinese Hamster Cell Mutants Deficient in Dihydrofolate Reductase Activity" Proc. Natl. Acad. Sci. USA vol. 77, No. 7, p. 4216-4220, 1980.
Van Dijk et al. "Human Antibodies as Next Generation Therapeutics" Current Opinion in Chem. Biol. 5:368-374, 2001.
Vaswani et al. "Humanized antibodies as potential therapeutic drugs", Ann Allergy Asthma Immunol. 81: 105-119, 1998.
Vaughan et al. "Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display libraby", Nature Biotechnology, vol. 14, pp. 309-314, 1996.
Verhoeyen et al. "Reshaping Human Antibodies: Grafting an Antilysozyme Activity", Science, 239:1534-1536, 1988.
Ward et al. "Binding activities of a repertoire of single immunoglobulin variable domains,secreted from *Escherichia coli*", Nature, vol. 341, pp. 544-546, 1989.
Waterhouse et al. "Combinatorial Infection and in Vivo Recombination: a Strategy for Making Large Phage Antibody Repertoires", Nuc. Acids. Res., 21:2265-2266, 1993.
Zhaolie, Chen :"Substitution of Asparagine for Glutamine for the Cultivation of Recombinant CHO Cell Line Producing Prourokinase"; Biotechnology Information, No. 2, pp. 25-27, published Feb. 28, 2001 (English translation).

* cited by examiner

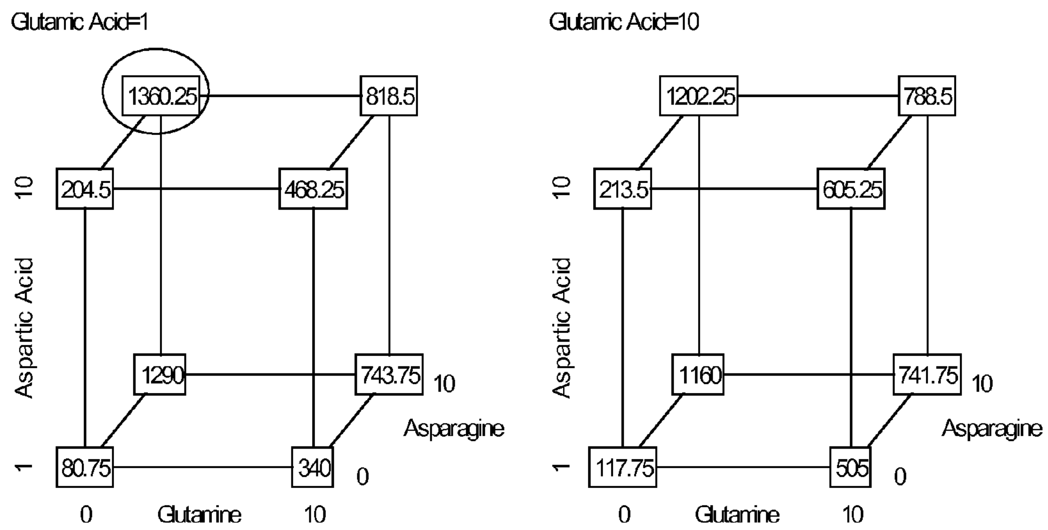


FIG. 1

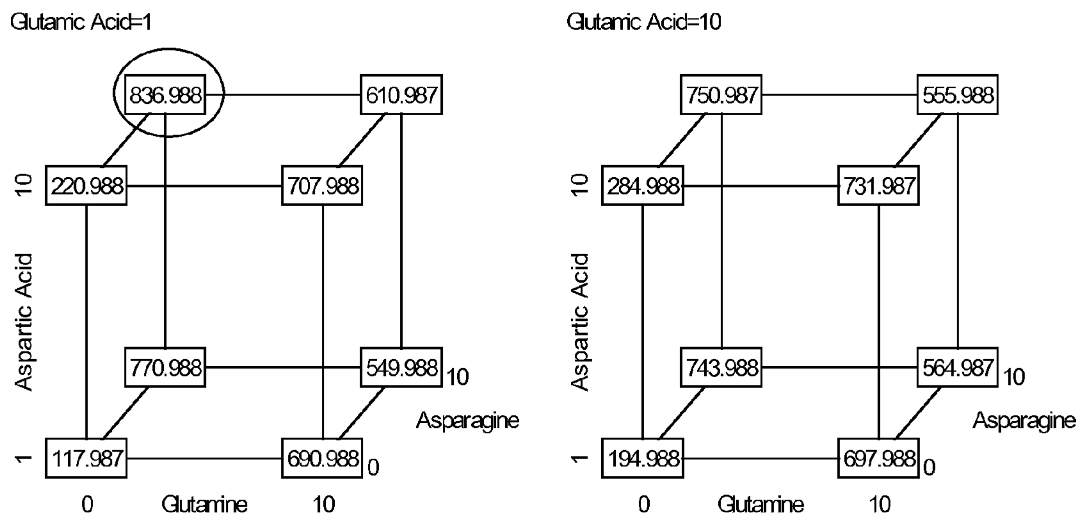


FIG. 2

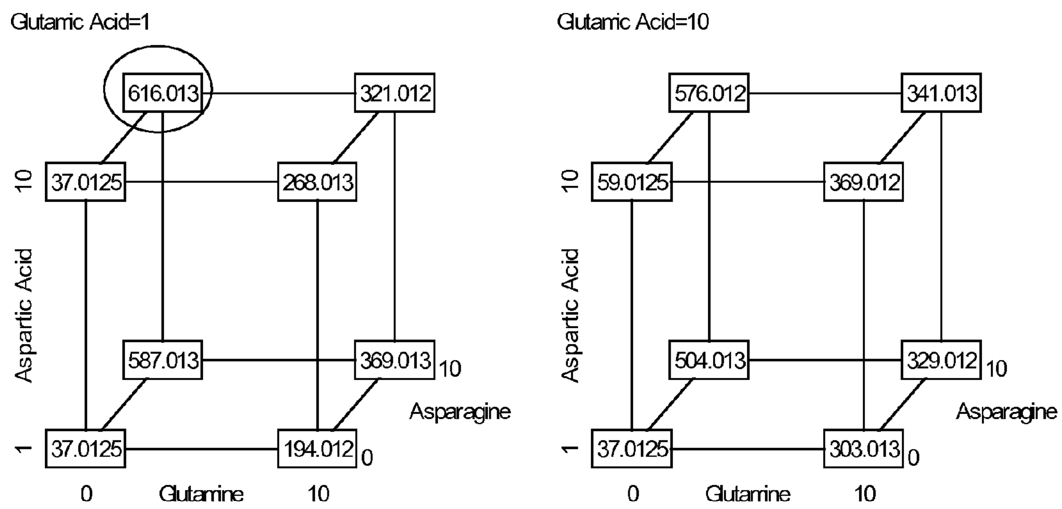


FIG. 3

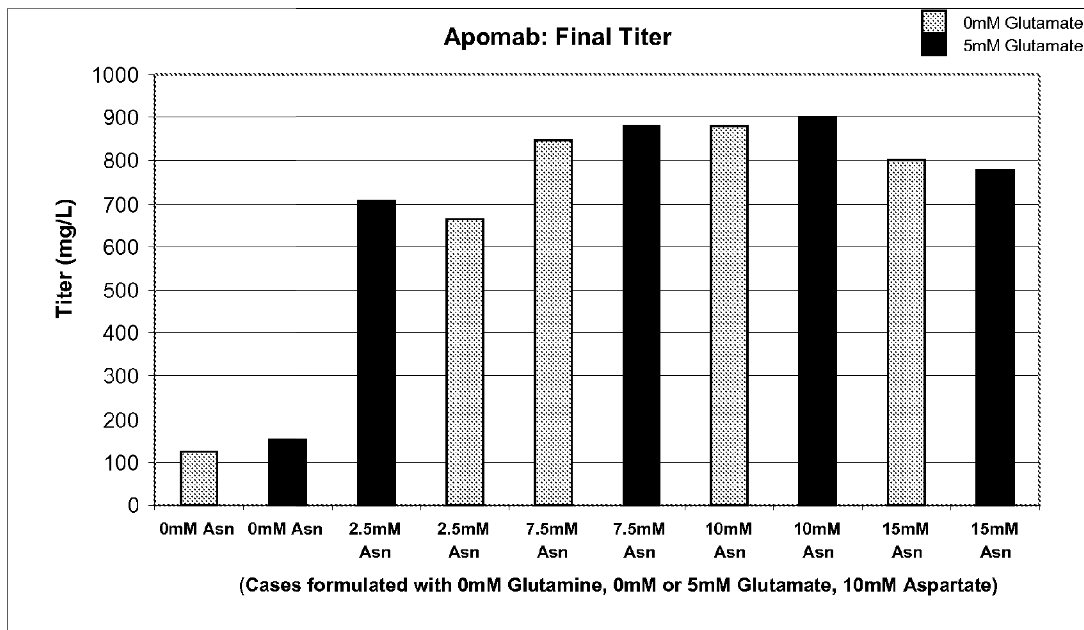
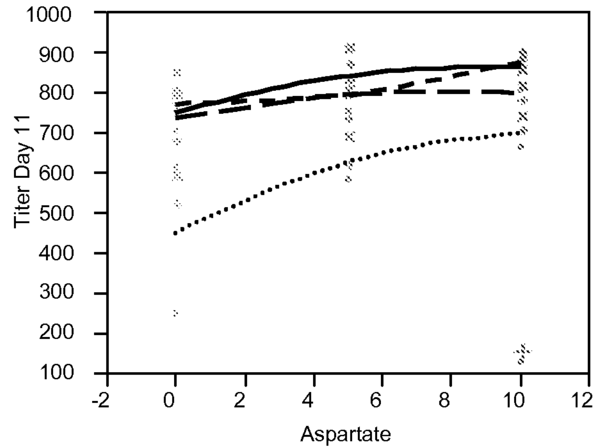


FIG. 4

Bivariate Fit of Titer Day 11 By Aspartate Glutamine=0



Smoothing Spline Fit, lambda=1 Asparagine==2.5
Smoothing Spline Fit, lambda=1 Asparagine==7.5
Smoothing Spline Fit, lambda=1 Asparagine==10
Smoothing Spline Fit, lambda=1 Asparagine==15

Smoothing Spline Fit, lambda=1 Asparagine==2.5

R-Square	0.573894
Sum of Squares Error	73461.63

Smoothing Spline Fit, lambda=1 Asparagine==7.5

R-Square	0.65596
Sum of Squares Error	11556.31

Smoothing Spline Fit, lambda=1 Asparagine==10

R-Square	0.408718
Sum of Squares Error	25684.65

Smoothing Spline Fit, lambda=1 Asparagine==15

R-Square	0.220438
Sum of Squares Error	26400.64

FIG. 5

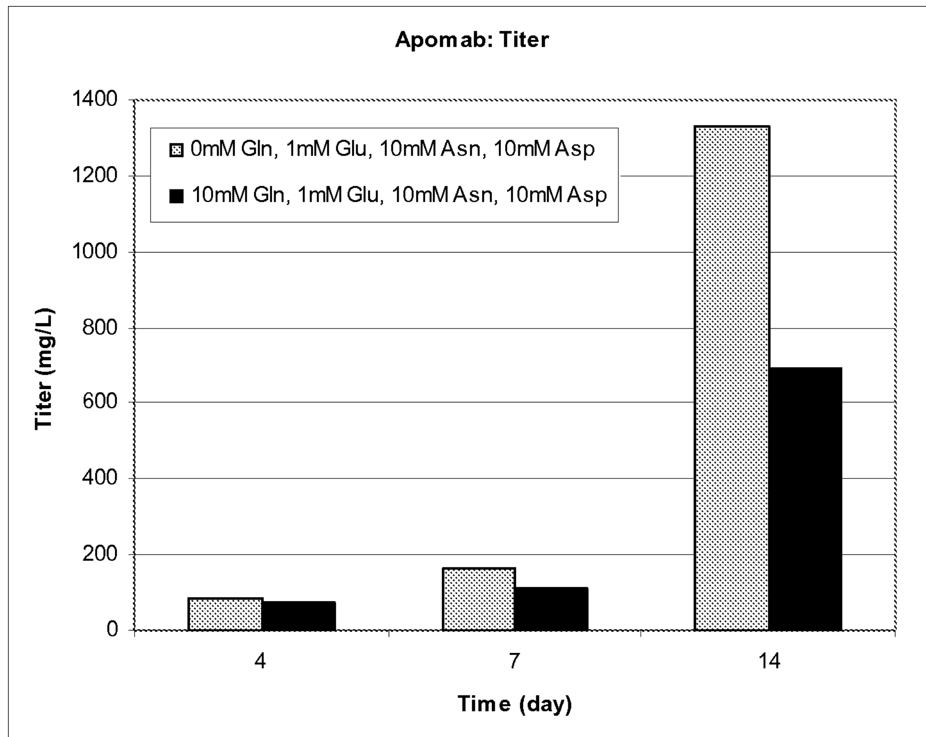


FIG. 6A

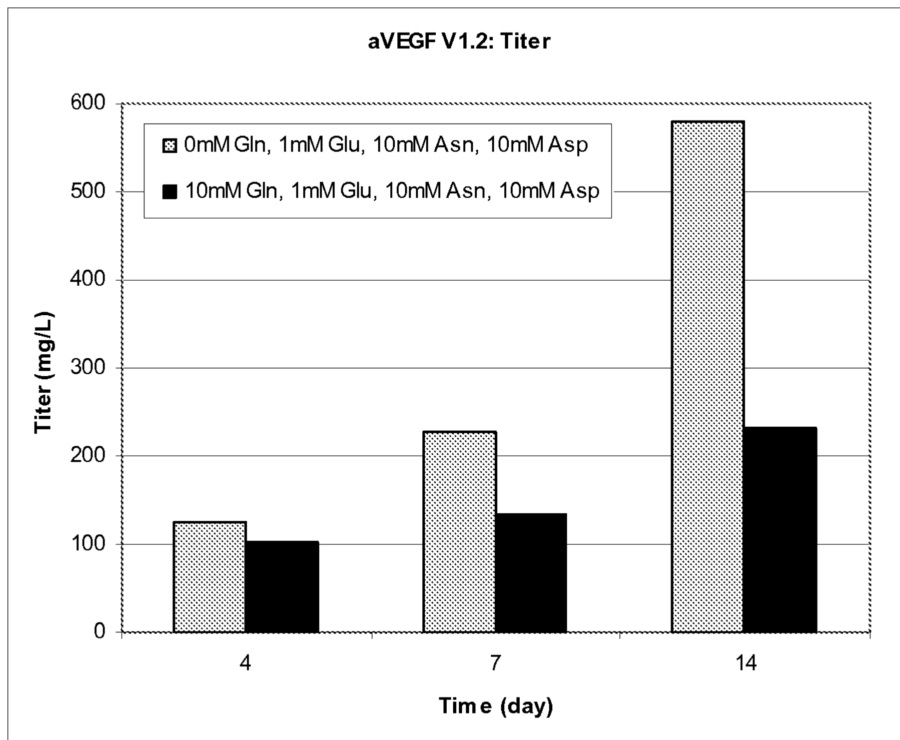


FIG. 6B

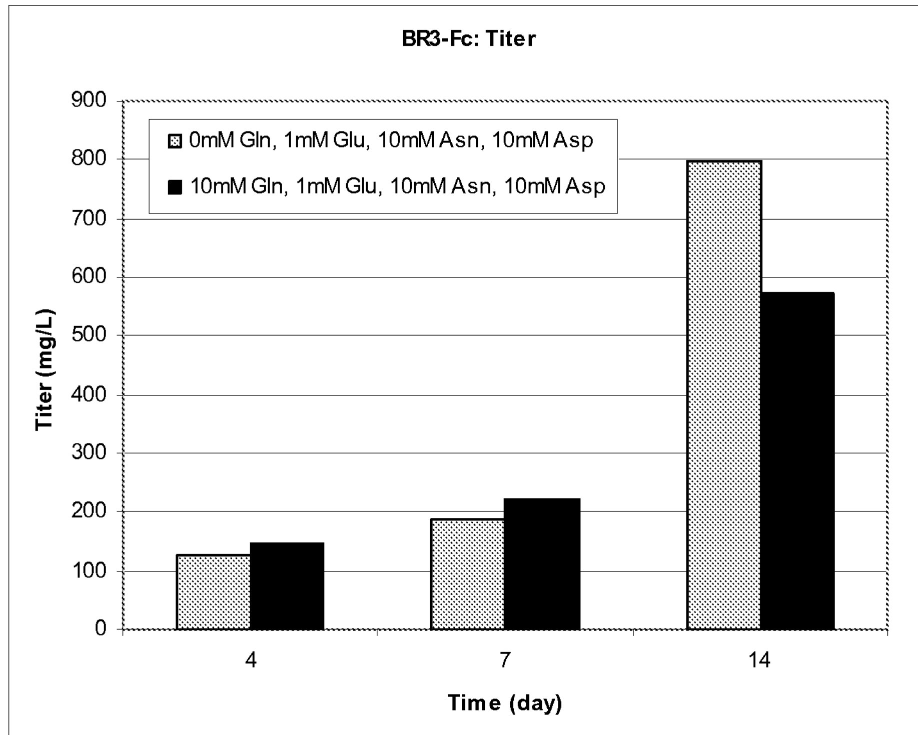


FIG. 6C

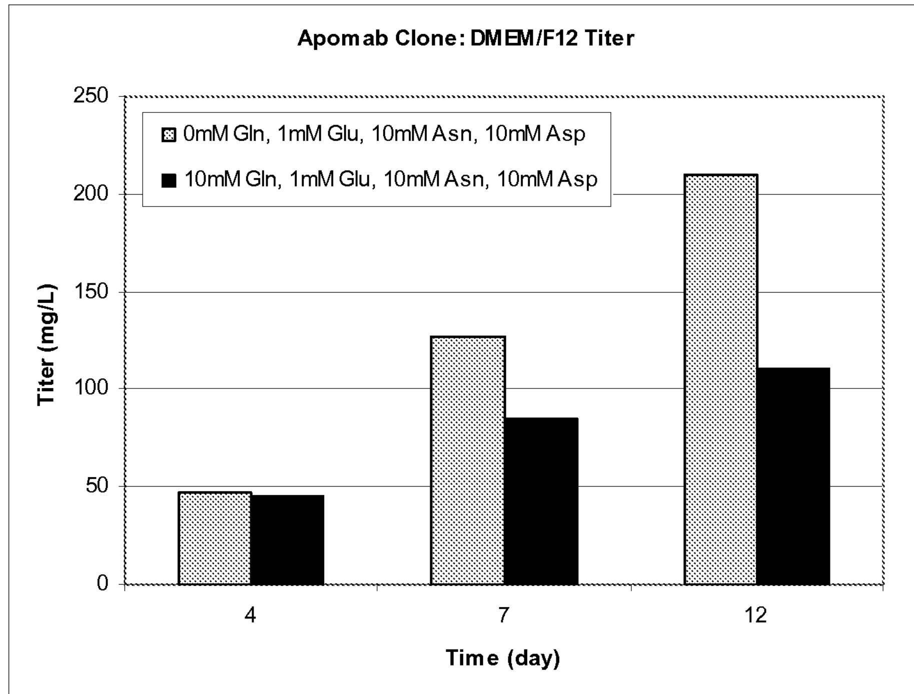


FIG. 7A

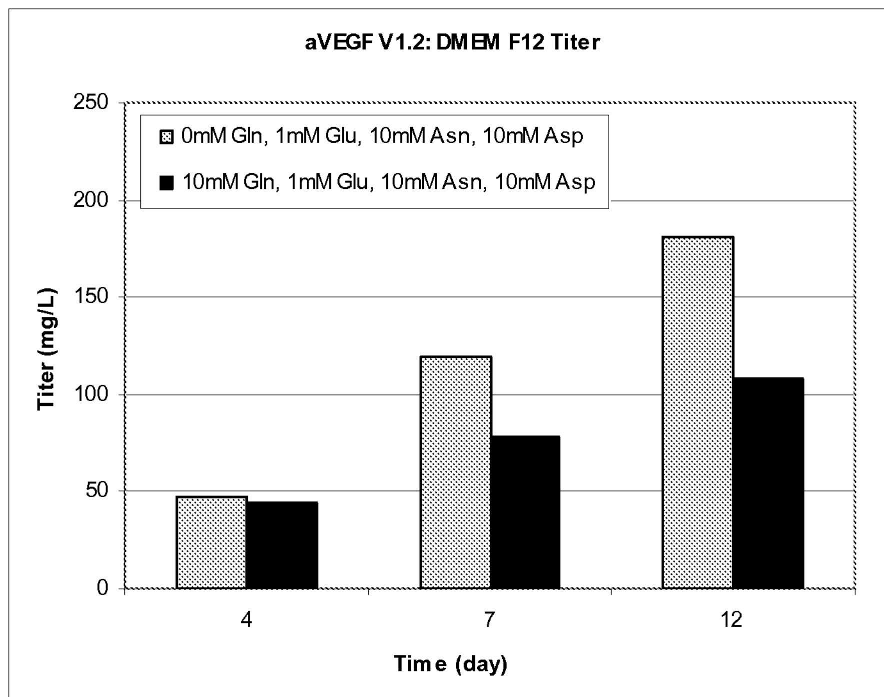


FIG. 7B

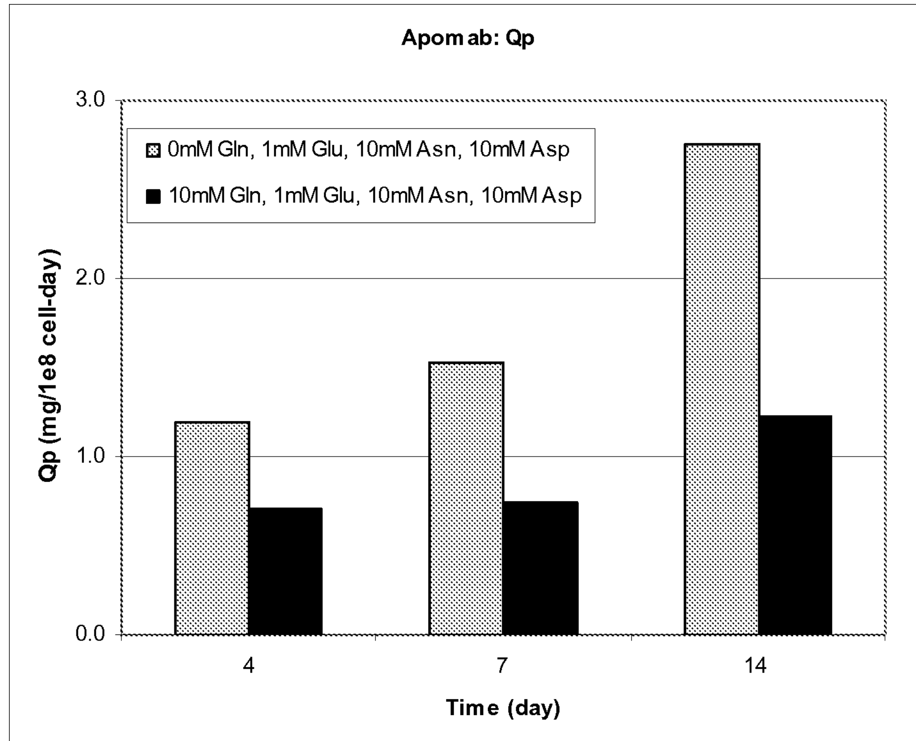


FIG. 8A

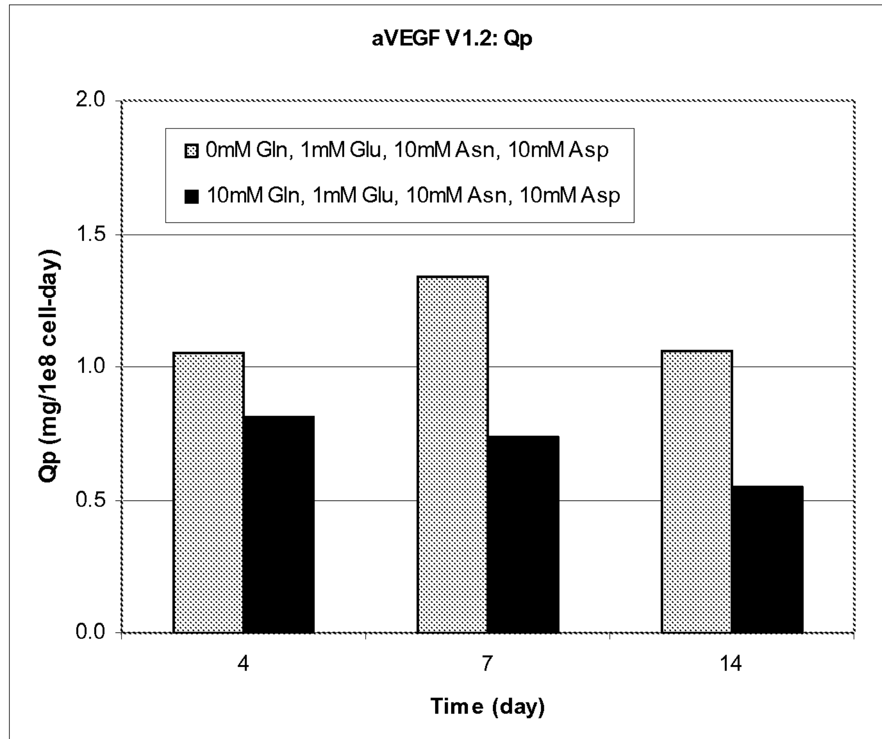


FIG. 8B

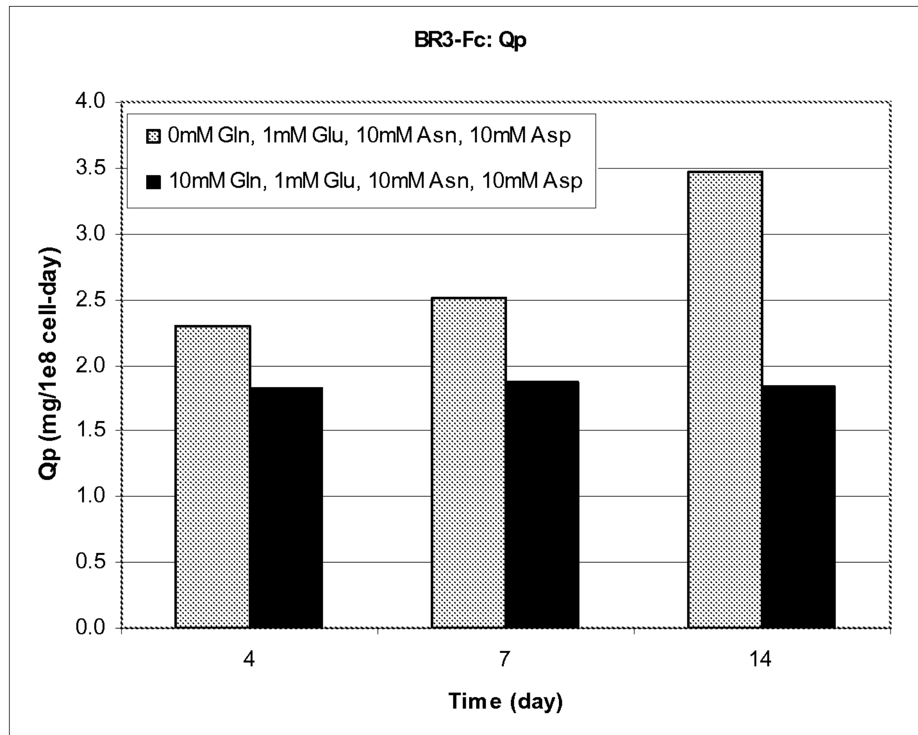


FIG. 8C

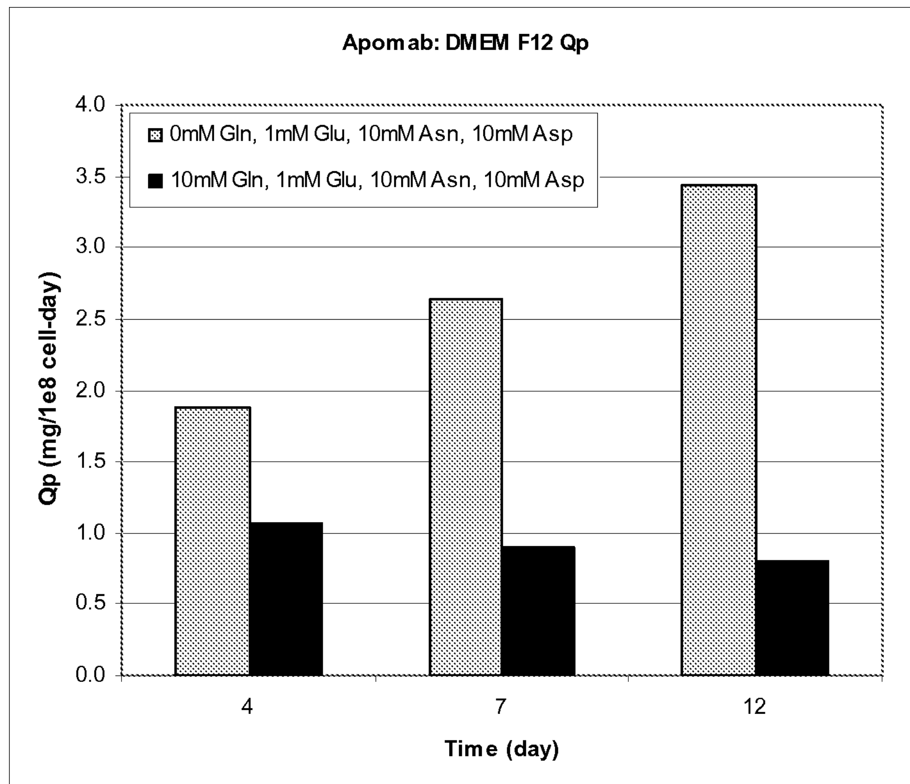


FIG. 9A

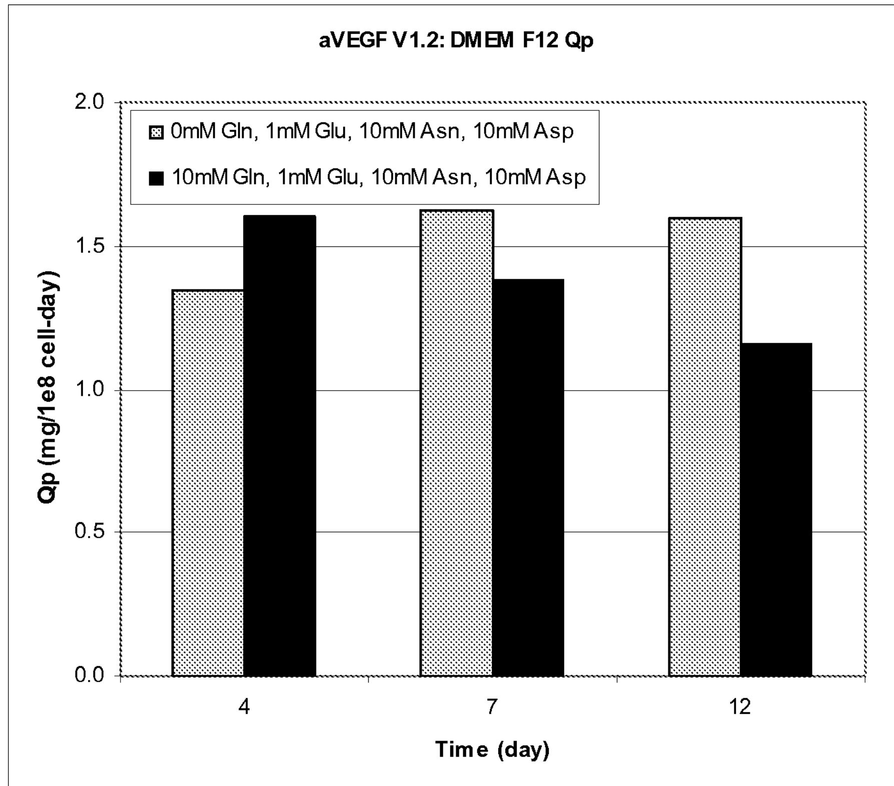


FIG. 9B

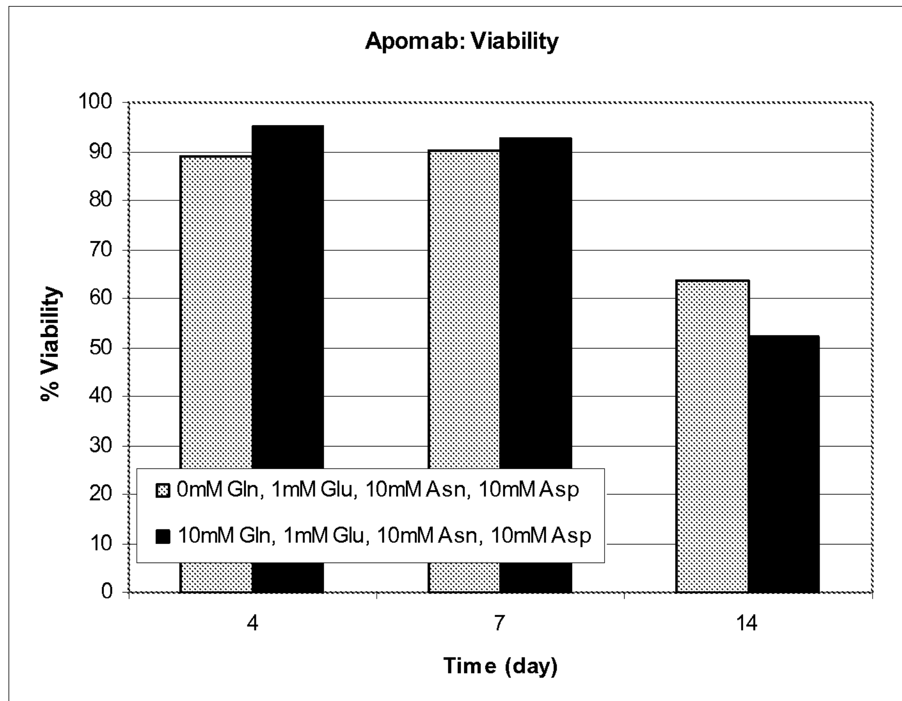


FIG. 10A

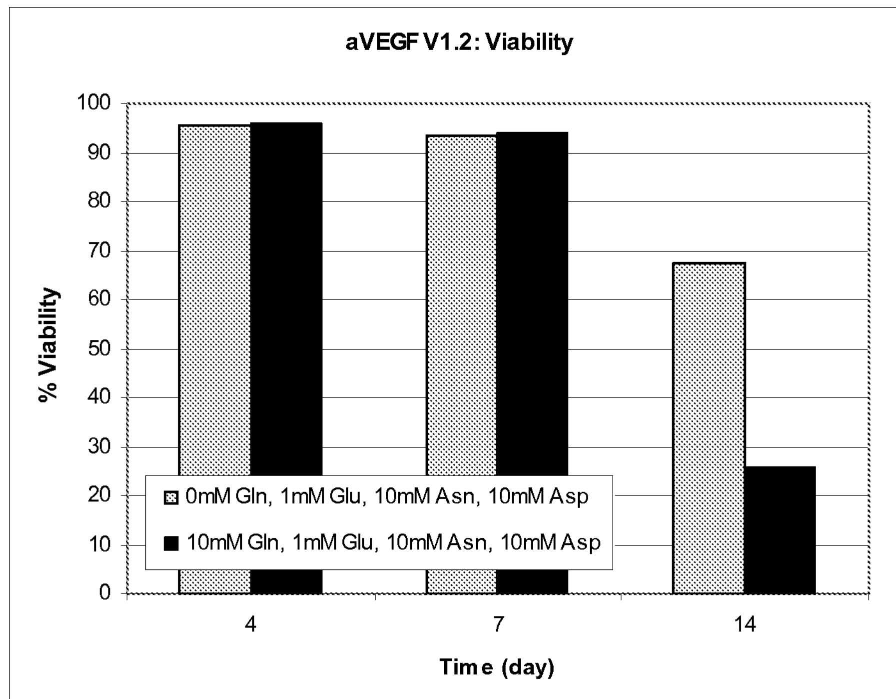


FIG. 10B

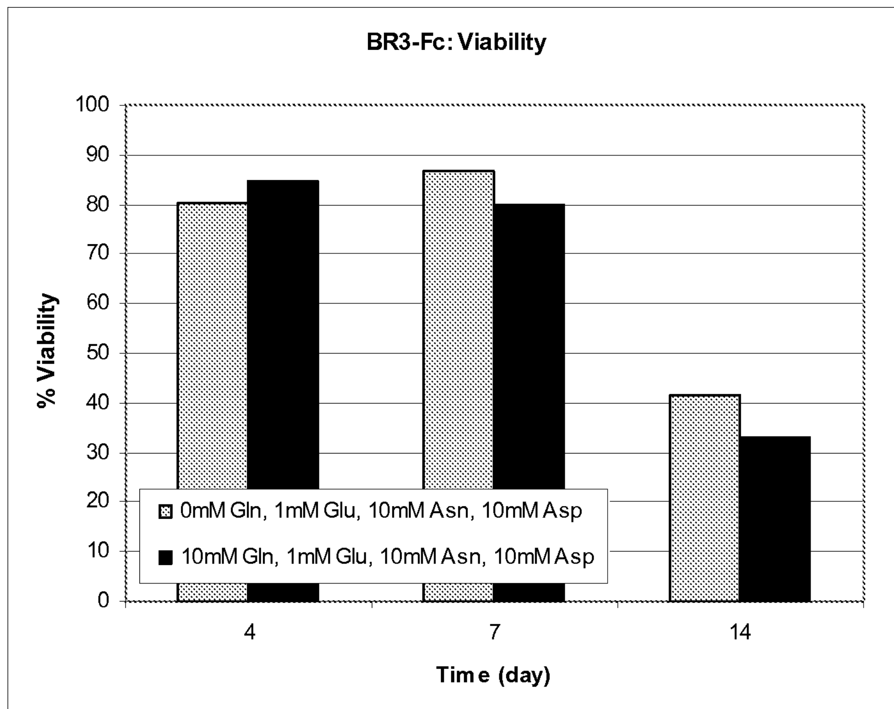


FIG. 10C

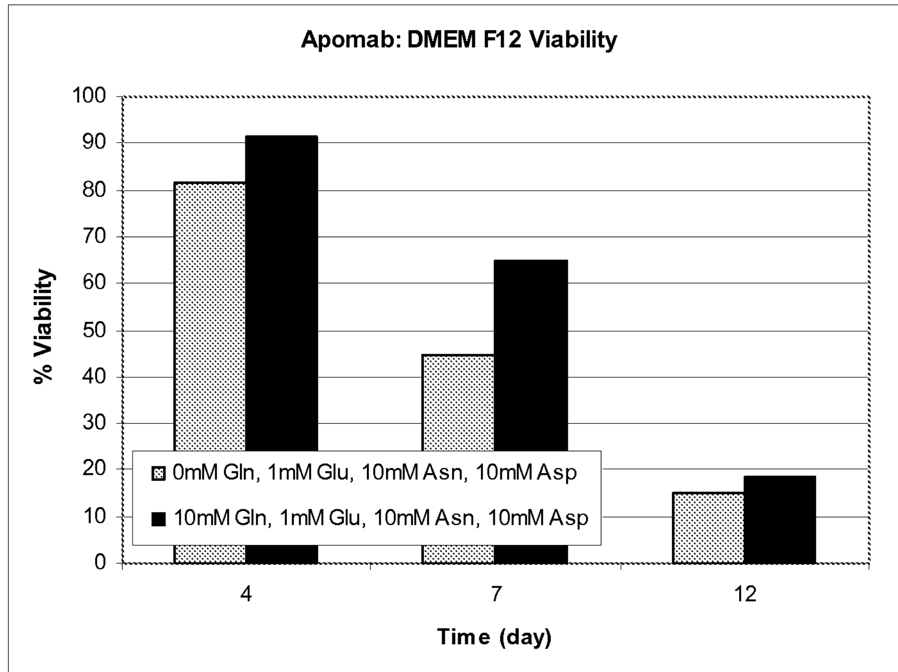


FIG. 11A

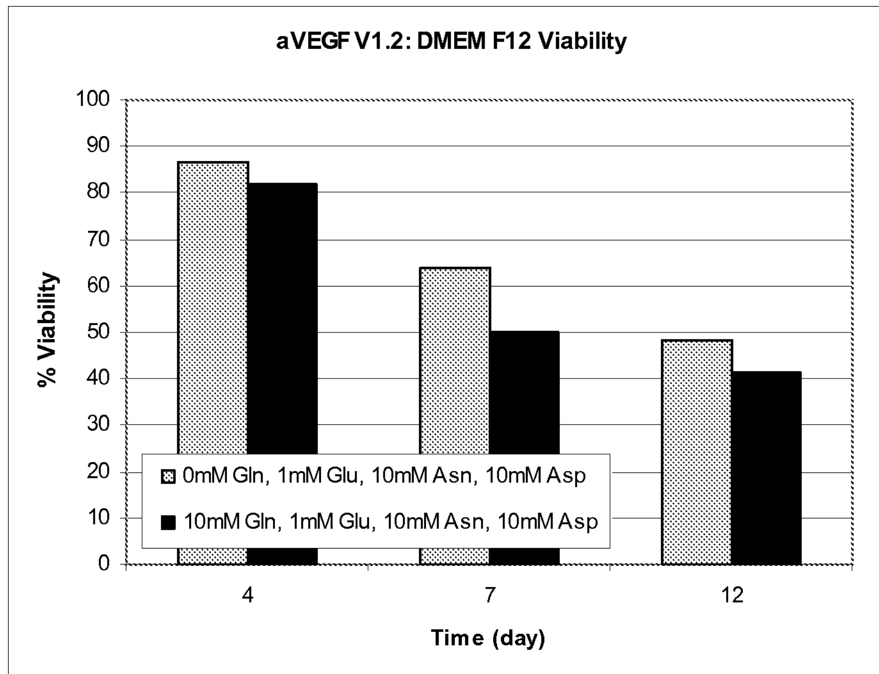


FIG. 11B

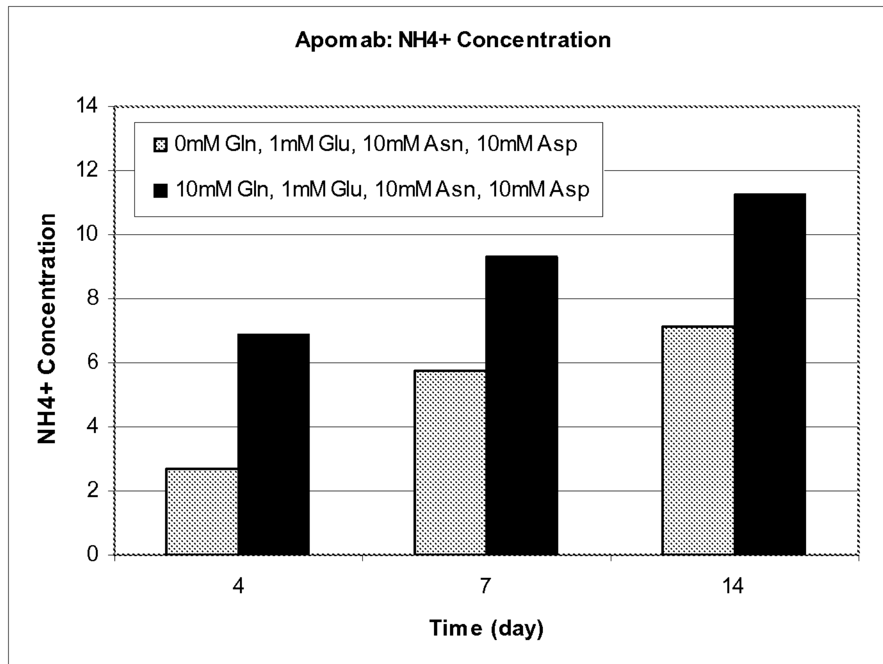


FIG. 12A

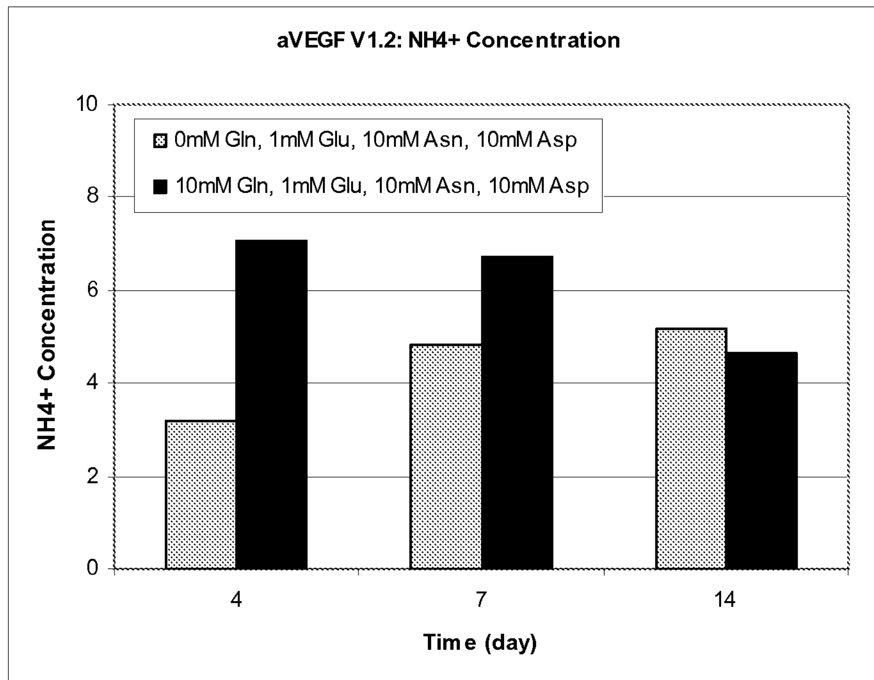


FIG. 12B

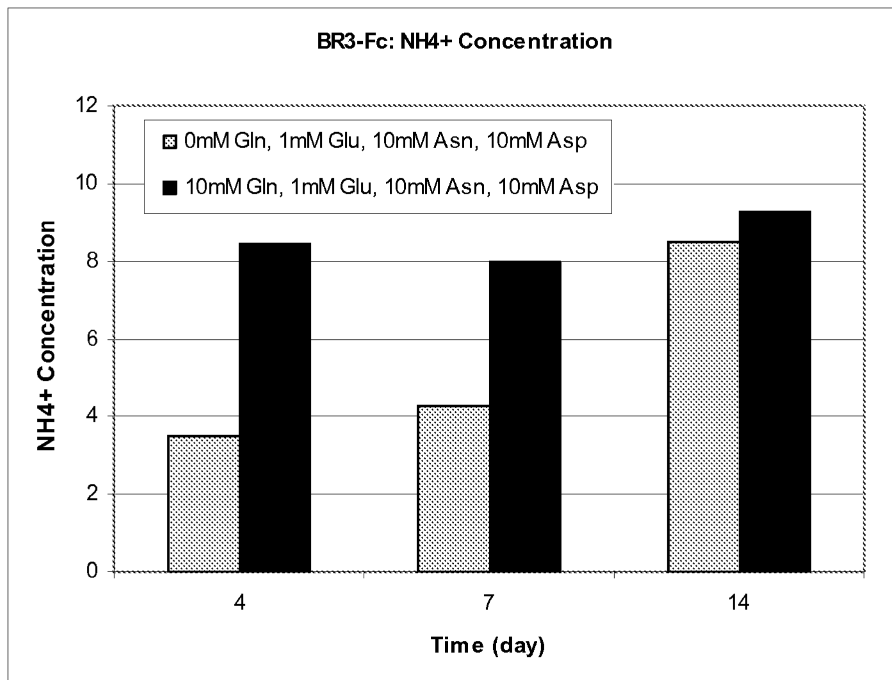


FIG. 12C

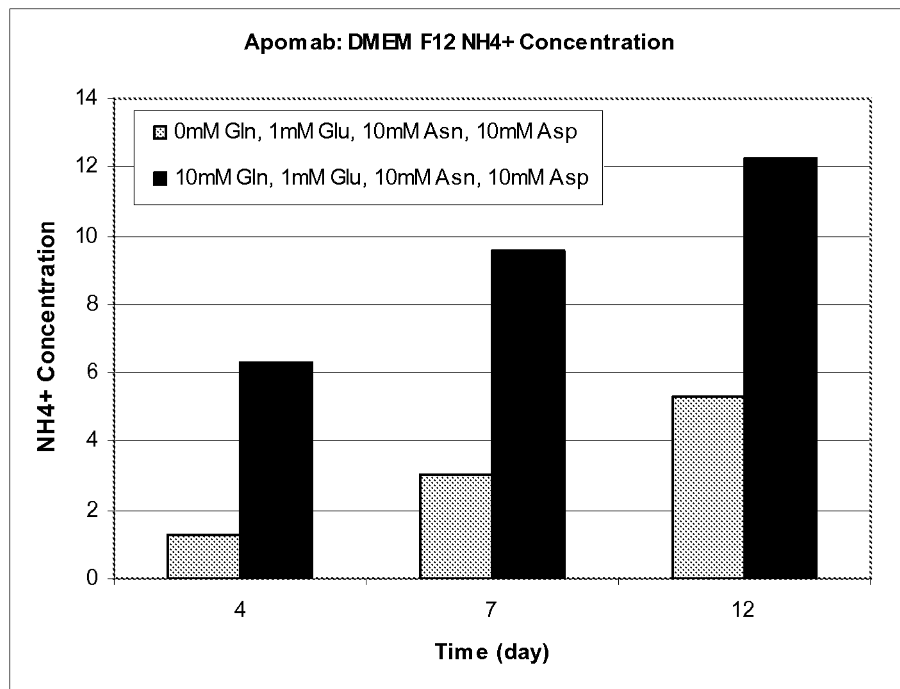


FIG. 13A

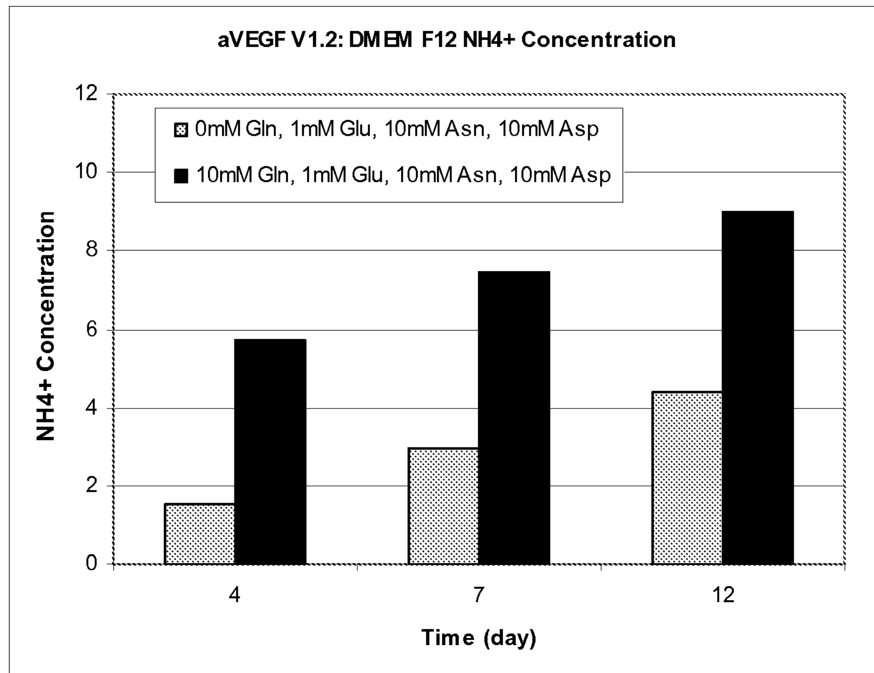


FIG. 13B

PRODUCTION OF PROTEINS IN GLUTAMINE-FREE CELL CULTURE MEDIA

RELATED APPLICATIONS

This application claims the benefit under 35 USC §119 to U.S. Provisional Application 61/232,889 filed Aug. 11, 2009.

FIELD OF THE INVENTION

The present invention relates generally to glutamine-free cell culture media. The invention further concerns the production of recombinant proteins, such as antibodies, in glutamine-free mammalian cell culture.

BACKGROUND OF THE INVENTION

Mammalian cells have become the dominant system for the production of mammalian proteins for clinical applications, primarily due to their ability to produce properly folded and assembled heterologous proteins, and their capacity for post-translational modifications. It is conventional to have glutamine in cell culture media during recombinant production of heterologous proteins, including antibodies. L-glutamine is an essential amino acid, which is considered the primary energy and nitrogen sources for cells in culture. Most commercially available media are formulated with free L-glutamine which is either included in the basal formula or added to liquid media formulations at the time of use. Thus, all mammalian cell culture media contain glutamine except those for glutamine synthetase transfected cell lines, such as GS NS0 and GS CHO cell lines, where the cells themselves produce the glutamine needed for growth. Glutamine is widely used at various concentrations typically from 1 to 20 mM in base media and much higher concentration in feeds for fed-batch process. For example, the concentration of L-glutamine is 0.5 mM in Ames' Medium and 10 mM in MCDP Media 131. DMEM/Ham's Nutrient Mixture F-12 (50:50) is often used as a starting formulation for proprietary media used with Chinese Hamster Ovary (CHO) cells. L-glutamine in DMEM/Ham's Nutrient Mixture F-12 is 2.5 mM. L-glutamine concentration in Serum-Free/Protein Free Hybridoma Medium is 2.7 mM. L-glutamine in DMEM, GMEM, IMDM and H-Y medium is 4 mM, of which IMDM is often used as a starting formulation for proprietary hybridoma cell culture media. It is generally held that hybridoma cells grow better in concentrations of L-glutamine that are above the average levels found in media. (Dennis R. Conrad, Glutamine in Cell Culture, Sigma-Aldrich Media Expert)

It was shown that glutamine is the main source of ammonia accumulated in cell culture (see review by Markus Schneider, et. al. 1996, *Journal of Biotechnology* 46:161-185). Thus, lowering glutamine in cell culture media significantly reduced the accumulation of NH_4^+ level, resulting in lower cytotoxicity (see Markus Schneider, et. al. 1996, supra). Reduced NH_4^+ cytotoxicity resulted in higher cell viability, thus extended culture longevity. Based on an estimated glutamine consumption study using CHO cells, it was suggested that cells may consume glutamine at a rate of 0.3-0.4 mM per day (Miller, et. al. 1988, Biotechnol. Bioeng. 32: 947-965). Altamirano et al. (2001, *J. Biotechnol.* 110:171-9) studied the effect of glutamine replacement by glutamate and the balance between glutamate and glucose metabolism on the redistribution of CHO cells producing recombinant human tissue plasminogen activator (rhut-PA). When glutamine was replaced with glutamate and balanced with glucose catabolism (carbon and nitrogen ratio, C/N ratio), cell metabolism was found redistributed and forced to utilize carbon and energy source more favorably to production of rhut-PA. It was also reported that CHO cells in adherent cultures can grow in the absence of added glutamine due to endogenous glutamine synthetase activity that allowed cells to synthesize glutamine from glutamic acid in the medium (Sanfeliu and Stephanopoulos, 1999, *Biotechnol. Bioeng.* 64:46-53). However, compared to control cultures in glutamine-containing media, the cell growth rate in glutamine-free media was slower with an increased fraction of cells distributed in the G0/G1 phase. The depletion of both glutamine and glutamic acid did cause cell death.

SUMMARY OF THE INVENTION

The present invention is based, at least in part, on the unexpected finding that not only can recombinant proteins be produced in a mammalian host cell using a glutamine-free production medium without any significant adverse effect, in fact the use of a glutamine-free medium in the production phase significantly increases cell viability, culture longevity, specific productivity and/or the final recombinant protein titer.

The present invention is also based on the unexpected finding that the addition of asparagine to a glutamine-free production medium can further enhance the cell viability, culture longevity, specific productivity and/or the final recombinant protein titer in a mammalian host cell using a glutamine-free production medium without any significant adverse effect.

In one aspect, the invention concerns a process for producing a polypeptide in a mammalian host cell expressing said polypeptide, comprising culturing the mammalian host cell in a production phase of the culture in a glutamine-free production culture medium supplemented with asparagine.

In one embodiment, the mammalian host cell is a Chinese Hamster Ovary (CHO) cell.

In another embodiment, the mammalian host cell is a $dhfr^-$ CHO cell.

In yet another embodiment, the production medium is serum-free.

In a further embodiment, the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

In a still further embodiment, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

In all embodiments, the production phase may, for example, be a batch or fed batch culture phase.

In all embodiments, the process may further comprise the step of isolating said polypeptide.

In a further embodiment, isolation may be followed by determining one or more of cell viability, culture longevity, specific productivity and final recombinant protein titer following isolation.

In a still further embodiment, at least one of the cell viability, culture longevity, specific productivity and final recom-

binant protein titer is increased relative to the same polypeptide produced in a glutamine-containing production medium of the same composition.

In a further aspect, the invention concerns a ready-to-use glutamine-free cell culture medium for the production of a polypeptide in a production phase.

In yet another embodiment, the polypeptide is a mammalian glycoprotein.

In other embodiments, the polypeptide is selected from the group consisting of antibodies, antibody fragments, and immunoadhesins.

In all embodiments, the polypeptide may, for example, be an antibody, or a biologically functional fragment of an antibody. Representative antibody fragments include Fab, Fab', $F(ab')_2$, scFv, $(scFv)_2$, dAb, complementarity determining region (CDR) fragments, linear antibodies, single-chain antibody molecules, minibodies, diabodies, and multispecific antibodies formed from antibody fragments.

In a still further embodiment, the antibody or antibody fragment is chimeric, humanized or human.

Therapeutic antibodies include, without limitation, anti-HER2 antibodies anti-CD20 antibodies; anti-IL-8 antibodies; anti-VEGF antibodies; anti-CD40 antibodies, anti-CD11a antibodies; anti-CD18 antibodies; anti-IgE antibodies; anti-Apo-2 receptor antibodies; anti-Tissue Factor (TF) antibodies; anti-human $a4\beta_7$ integrin antibodies; anti-EGFR antibodies; anti-CD3 antibodies; anti-CD25 antibodies; anti-CD4 antibodies; anti-CD52 antibodies; anti-Fc receptor antibodies; anti-carcinoembryonic antigen (CEA) antibodies; antibodies directed against breast epithelial cells; antibodies that bind to colon carcinoma cells; anti-CD38 antibodies; anti-CD33 antibodies; anti-CD22 antibodies; anti-EpCAM antibodies; anti-GpIIb/IIIa antibodies; anti-RSV antibodies; anti-CMV antibodies; anti-HIV antibodies; anti-hepatitis antibodies; anti-CA 125 antibodies; anti-$\alpha v\beta 3$ antibodies; anti-human renal cell carcinoma antibodies; anti-human 17-1A antibodies; anti-human colorectal tumor antibodies; anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma; and anti-human leukocyte antigen (HLA) antibodies, and anti-HLA DR antibodies.

In other embodiments, the therapeutic antibody is an antibody binding to a HER receptor, VEGF, IgE, CD20, CD11a, CD40, or DR5.

In other embodiments, the therapeutic antibody is an anti-BR3 antibody or BR3-Fc immunoadhesin.

In other embodiments of the method of the present invention, the polypeptide expressed in the recombinant host cell is a therapeutic polypeptide. For example, the therapeutic polypeptide can be selected from the group consisting of a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD 18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of said polypeptides.

In all embodiments, the recombinant host cell can be an eukaryotic host cell. such as a mammalian host cell, including, for example, Chinese Hamster Ovary (CHO) cells.

These and other aspects will be apparent from the description below, including the Examples and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Apomab antibody cube plot analysis of titer results from a Full Factorial Design of Experiment (DOE) evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate. The model predicts that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

FIG. **2**. BR3-Fc immunoadhesin cube plot analysis of titer results from a Full Factorial DOE evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate. The model predicts that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

FIG. **3**. anti-VEGF antibody cube plot analysis of titer results from a Full Factorial DOE evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate. The model predicts that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

FIG. **4**. Effect of Asparagine under Glutamine-free, low Glutamate and high Aspartate conditions on Apomab antibody titer. In Glutamine-free medium, Apomab antibody titer was significantly increased in the presence of 2.5-15 mM Asparagine compared to Glutamine-free cultures without Asparagine. Under these conditions, the presence or absence of Glutamate had no effect on titer.

FIG. **5**. Apomab antibody titer production across various Asparagine and Aspartate concentrations in Glutamine-free

and low Glutamate conditions. A positive titration effect was observed when increasing Aspartate from 0 to 10 mM under these conditions.

FIGS. **6**. A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on titer. The final titer for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin was significantly higher in Glutamine-free medium compared to Glutamine-containing medium.

FIGS. **7**A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on titer. The final titer for Apomab antibody and anti-VEGF antibody was significantly higher in Glutamine-free DMEM/F12 medium compared to Glutamine-containing DMEM F12 medium.

FIGS. **8**A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on cell specific productivity (Qp). Cell specific productivity for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin was significantly higher in Glutamine-free medium compared to Glutamine-containing medium.

FIGS. **9**A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on cell specific productivity (Qp). Cell specific productivity for Apomab antibody and anti-VEGF antibody was significantly higher in Glutamine-free DMEM/F12 medium compared to Glutamine-containing DMEM/F12 medium.

FIGS. **10**A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on Cell Viability. Cell viability for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin was higher in Glutamine-free medium compared to Glutamine-containing medium.

FIGS. **11**A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on Cell Viability. In DMEM/F12 medium, cell viability was not consistently improved in Glutamine-free medium. Viability was higher for Apomab antibody, but lower for anti-VEGF antibody compared to Glutamine containing medium.

FIGS. **12** A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on ammonia formation Ammonia was usually lower in Glutamine-free cultures compared to Glutamine-containing cultures.

FIGS. **13**A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on ammonia formation. Ammonia was significantly reduces in Glutamine-free DMEM/F12 medium compared to Glutamine-containing DMEM/F12 medium.

DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

The terms "cell culture medium", "culture medium", and "nutrient mixture" refer to a nutrient solution used for growing mammalian cells that typically provides at least one component from one or more of the following categories:

1) an energy source, usually in the form of a carbohydrate such as glucose;

2) some or all of the essential amino acids, and usually the basic set of twenty amino acids plus cystine;

3) vitamins and/or other organic compounds typically required at low concentrations;

4) free fatty acids; and

5) trace elements, where trace elements are defined as inorganic compounds or naturally occurring elements that are typically required at very low concentrations, usually in the micromolar range.

The nutrient mixture may optionally be supplemented with one or more component from any of the following categories:

1) hormones and other growth factors as, for example, insulin, transferrin, and epidermal growth factor;

2) salts and buffers as, for example, calcium, magnesium, and phosphate; and

3) nucleosides such as, for example, adenosine and thymidine.

The cell culture medium is generally "serum free" when the medium is essentially free of serum from any mammalian source (e.g. fetal bovine serum (FBS)). By "essentially free" is meant that the cell culture medium comprises between about 0-5% serum, preferably between about 0-1% serum, and most preferably between about 0-0.1% serum. Advantageously, serum-free "defined" medium can be used, wherein the identity and concentration of each of the components in the medium is known (i.e., an undefined component such as bovine pituitary extract (BPE) is not present in the culture medium).

In the context of the present invention the expressions "cell", "cell line", and "cell culture" are used interchangeably, and all such designations include progeny. Thus, the words "transformants" and "transformed (host) cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

The term "animal host cell," "animal cell," "animal recombinant host cell," and the like, encompasses invertebrate, non-mammalian vertebrate (e.g., avian, reptile and amphibian) and mammalian cells. Examples of invertebrate cells include the following insect cells: *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori*. See, e.g., Luckow et al., Bio/Technology, 6:47-55 (1988); Miller et al., in *Genetic Engineering*, Setlow, J. K. et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., *Nature,* 315:592-594 (1985).

The term "mammalian host cell," "mammalian cell," "mammalian recombinant host cell," and the like, refer to cell lines derived from mammals that are capable of growth and survival when placed in either monolayer culture or in suspension culture in a medium containing the appropriate nutrients and growth factors. The necessary nutrients and growth factors for a particular cell line are readily determined empirically without undue experimentation, as described for example in *Mammalian Cell Culture* (Mather, J. P. ed., Plenum Press, N. Y. (1984)), and by Barnes and Sato (*Cell,* 22:649 (1980)). Typically, the cells are capable of expressing and secreting large quantities of a particular protein of interest (typically a recombinant protein) into the culture medium, and are cultured for this purpose. However, the cells may be cultured for a variety of other purposes as well, and the scope of this invention is not limited to culturing the cells only for production of recombinant proteins. Examples of suitable mammalian cell lines, capable of growth in the media of this

invention, include monkey kidney CVI line transformed by SV40 (COS-7, ATCC® CRL 1651); human embryonic kidney line 293S (Graham et al., *J. Gen. Virolo.,* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC® CCL 10); mouse sertoli cells (TM4, Mather, *Biol. Reprod.,* 23:243 (1980)); monkey kidney cells (CVI-76, ATCC® CCL 70); African green monkey kidney cells (VERO-76, ATCC® CRL-1587); human cervical carcinoma cells (HELA, ATCC® CCL 2); canine kidney cells (MDCK, ATCC® CCL 34); buffalo rat liver cells (BRL 3A, ATCC® CRL 1442); human lung cells (W138, ATCC® CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor cells (MMT 060562, ATCC® CCL 5I); rat hepatoma cells (HTC, MI.54, Baumann et al., J. Cell Biol., 85:1 (1980)); and TR-1 cells (Mather et al., *Annals N.Y. Acad. Sci.,* 383:44 (1982)) and hybridoma cell lines. Chinese hamster ovary cells (Urlab and Chasin, *Proc. Natl. Acad. Sci. USA,* 77:4216 (1980)) are a preferred cell line for practicing this invention. CHO cells suitable for use in the methods of the present invention have also been described in the following documents: EP 117,159, published Aug. 29, 1989; U.S. Pat. Nos. 4,766,075; 4,853,330; 5,185,259; Lubiniecki et al., in *Advances in Animal Cell Biology and Technology for Bioprocesses*, Spier et al., eds. (1989), pp. 442-451. Known CHO derivatives suitable for use herein include, for example, CHO/-DHFR (Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA,* 77: 4216 (1980)), CHO-K1 DUX B11 (Simonsen and Levinson, *Proc. Natl. Acad. Sci. USA* 80: 2495-2499 (1983); Urlaub and Chasin, supra), and dp 12.CHO cells (EP 307,247 published Mar. 15, 1989). Preferred host cells include CHO-K1 DUX B11 and dp 12.CHO cells.

"dhfr$^-$ CHO cell" refers to a dihydrofolate reductase (DHFR) deficient CHO cell. Production of recombinant proteins in mammalian cells has allowed the manufacture of a number of large, complex glycosylated polypeptides for clinical applications. Chinese hamster ovary (CHO) DHFR– cells and the amplifiable selectable marker DHFR are routinely used to establish cell lines that produce clinically useful amounts of product. (Urlab, G. and Chasin, L. A. (1980) Proc. Natl Acad. Sci. USA, 77, 4216-4220; Kaufman, R. J. and Sharp, P. (1982) J. Mol. Biol., 159, 601-621; Gasser, C. S., Simonsen, C. S., Schilling, J. W. and Schmike, R. T. (1982) Proc. Natl Sci. USA, 79, 6522-6526)

By "phase" is meant a certain phase of culturing of the cells as is well recognized by the practitioner.

"Growth phase" of the cell culture refers to the period of exponential cell growth (the log phase) where cells are generally rapidly dividing. During this phase, cells are cultured for a period of time, usually between 1-4 days, and under such conditions that cell growth is maximized. The growth cycle for the host cell can be determined for the particular host cell envisioned without undue experimentation. During the growth phase, cells are cultured in nutrient medium containing the necessary additives generally at about 30-40° C., preferably about 37° C., in a humidified, controlled atmosphere, such that optimal growth is achieved for the particular cell line. Cells are maintained in the growth phase for a period of between about one and four days, usually between about two and three days.

"Transition phase" of the cell culture refers to the period of time during which culture conditions for the production phase are engaged. During the transition phase environmental factors such as temperature are shifted from growth conditions to production conditions.

"Production phase" of the cell culture refers to the period of time during which cell growth has plateaued. During the production phase, logarithmic cell growth has ended and protein production is primary. During this period of time the medium is generally supplemented to support continued protein production and to achieve the desired protein product.

The phrase "fed batch cell culture" when used herein refers to a batch culture wherein the animal (e.g. mammalian) cells and culture medium are supplied to the culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. Fed batch culture includes "semi-continuous fed batch culture" wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium. Fed batch culture is distinguished from simple "batch culture" in which all components for cell culturing (including the animal cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed batch culture can be further distinguished from perfusion culturing insofar as the supernatant is not removed from the culturing vessel during the process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers etc and the culture medium is continuously or intermittently introduced and removed from the culturing vessel). However, removal of samples for testing purposes during fed batch cell culture is contemplated.

When used herein, the term "glutamine" refers to the amino acid L-glutamine (also known as "Gln" and "Q" by three-letter and single-letter designation, respectively) which is recognized as both an amino acid building block for protein synthesis and as an energy source in cell culture. Thus, the terms "glutamine" and "L-glutamine" are used interchangeably herein.

The word "glucose" refers to either of α-D-glucose or β-D-glucose, separately or in combination. It is noted that α and β glucose forms are interconvertible in solution.

The expression "osmolality" is a measure of the osmotic pressure of dissolved solute particles in an aqueous solution. The solute particles include both ions and non-ionized molecules. Osmolality is expressed as the concentration of osmotically active particles (i.e., osmoles) dissolved in 1 kg of water (1 mOsm/kg H_2O at 38° C. is equivalent to an osmotic pressure of 19 mm Hg). "Osmolarity" refers to the number of solute particles dissolved in 1 liter of solution. Solutes which can be added to the culture medium so as to increase the osmolality thereof include proteins, peptides, amino acids, non-metabolized polymers, vitamins, ions, salts, sugars, metabolites, organic acids, lipids, etc. In the preferred embodiment, the concentration of amino acids and NaCl in the culture medium is increased in order to achieve the desired osmolality ranges set forth herein. When used herein, the abbreviation "mOsm" means "milliosmoles/kg H_2O".

The term "cell density" as used herein refers to that number of cells present in a given volume of medium.

The term "cell viability" as used herein refers to the ability of cells in culture to survive under a given set of culture conditions or experimental variations. The term as used herein also refers to that portion of cells which are alive at a particular time in relation to the total number of cells, living and dead, in the culture at that time.

The terms "amino acids" and "amino acid" refer to all naturally occurring alpha amino acids in both their D and L stereoisomeric forms, and their analogs and derivatives. An analog is defined as a substitution of an atom in the amino acid with a different atom that usually has similar properties. A derivative is defined as an amino acid that has another molecule or atom attached to it. Derivatives would include, for

example, acetylation of an amino group, amination of a carboxyl group, or oxidation of the sulfur residues of two cysteine molecules to form cystine.

The term "protein" is meant to refer to a sequence of amino acids for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. This is to distinguish from "peptides" or other small molecular weight drugs that do not have such structure. Typically, the protein herein will have a molecular weight of at least about 15-20 kD, preferably at least about 20 kD. Examples of proteins encompassed within the definition herein include all mammalian proteins, in particular, therapeutic and diagnostic proteins, such as therapeutic and diagnostic antibodies, and, in general proteins that contain one or more disulfide bonds, including multi-chain polypeptides comprising one or more inter- and/or intrachain disulfide bonds.

The term "therapeutic protein" or "therapeutic polypeptide" refers to a protein that is used in the treatment of disease, regardless of its indication or mechanism of action. In order for therapeutic proteins to be useful in the clinic it must be manufactured in large quantities. "Manufacturing scale" production of therapeutic proteins, or other proteins, utilize cell cultures ranging from about 400 L to about 80,000 L, depending on the protein being produced and the need. Typically such manufacturing scale production utilizes cell culture sizes from about 400 L to about 25,000 L. Within this range, specific cell culture sizes such as 4,000 L, about 6,000 L, about 8,000, about 10,000, about 12,000 L, about 14,000 L, or about 16,000 L are utilized.

As used herein, "polypeptide of interest" refers generally to peptides and proteins having more than about ten amino acids. The polypeptides may be homologous to the host cell, or preferably, may be exogenous, meaning that they are heterologous, i.e., foreign, to the host cell being utilized, such as a human protein produced by a non-human mammalian, e.g., Chinese Hamster Ovary (CHO) cell. Preferably, mammalian polypeptides (polypeptides that were originally derived from a mammalian organism) are used, more preferably those which are directly secreted into the medium. The term "polypeptide" or "polypeptide of interest" specifically includes antibodies, in particular, antibodies binding to mammalian polypeptides, such as any of the mammalian polypeptides listed below or fragments thereof, as well as immunoadhesins (polypeptide-Ig fusion), such as those comprising any of the mammalian polypeptides listed below, or fragments thereof.

Examples of mammalian polypeptides include, without limitation, transmembrane molecules (e.g. receptors) and ligands such, as growth factors. Exemplary polypeptides include molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; interferon such as interferon-α, -β, and -γ; lipoproteins; α-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA), including t-PA variants; bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as β-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER1 (EGFR), HER2, HER3 or HER4 receptor; Apo2L/TRAIL, hedgehog, mitogen activated protein kinase (MAPK), and fragments of any of the above-listed polypeptides. Apo2L (TRAIL) and is variants are disclosed, for example, in U.S. Application Publication No. 20040186051. Anti-VEGF antibodies are disclosed, for example, in U.S. Pat. Nos. 8,994,879; 7,060,269; 7,169,901; and 7,297,334. Anti-CD20 antibodies are disclosed, for example, in U.S. Application Publication No. 20060246004. The BR3 polypeptide, anti-BR3 antibodies and BR3-Fc immunoadhesins are described, for example, in U.S. Application Publication No. 20050070689.

As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the binding specificity of a heterologous protein (an "adhesin") with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of an amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site of an antibody (i.e., is "heterologous"), and an immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding site of a receptor or a ligand. The immunoglobulin constant domain sequence in the immunoadhesin may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA (including IgA-1 and IgA-2), IgE, IgD or IgM.

As noted above, in certain embodiments, the protein is an antibody. "Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which generally lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region or intact monoclonal antibodies), antibody compositions with polyepitopic specificity, polyclonal antibodies, multivalent antibodies, multispecific antibodies (e.g., bispecific antibod-

ies) formed from at least two intact antibodies, diabodies, and single-chain molecules such as scFv molecules, as well as antibody fragments (e.g., Fab, F(ab')2, and Fv).

Unless indicated otherwise, the expression "multivalent antibody" is used throughout this specification to denote an antibody comprising three or more antigen binding sites. The multivalent antibody is typically engineered to have the three or more antigen binding sites and is generally not a native sequence IgM or IgA antibody.

The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain the Fc region.

"Antibody fragments" comprise only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such an antibody fragment may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fe" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')2 fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')2 antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known. Examples of antibody fragments encompassed by the present definition include: (i) the Fab fragment, having VL, CL, VH and CH1 domains; (ii) the Fab' fragment, which is a Fab fragment having one or more cysteine residues at the C-terminus of the CH1 domain; (iii) the Fd fragment having VH and CH1 domains; (iv) the Fd' fragment having VH and CH1 domains and one or more cysteine residues at the C-terminus of the CH1 domain; (v) the Fv fragment having the VL and VH domains of a single arm of an antibody; (vi) the dAb fragment (Ward et al., *Nature* 341, 544-546 (1989)) which consists of a VH domain; (vii) isolated CDR regions; (viii) F(ab')2 fragments, a bivalent fragment including two Fab' fragments linked by a disulphide bridge at the hinge region; (ix) single chain antibody molecules (e.g. single chain Fv; scFv) (Bird et al., *Science* 242: 423-426 (1988); and Huston et al., *PNAS* (*USA*) 85:5879-5883 (1988)); (x) "diabodies" with two antigen binding sites, comprising a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (see, e.g., EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993)); (xi) "linear antibodies" comprising a pair of tandem Fd segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata et al. *Protein Eng.* 8(10):1057 1062 (1995); and U.S. Pat. No. 5,641,870).

"Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

"Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404,097; W093/1161; Hudson et al., (2003) Nat. Med. 9:129-134; and Hollinger et al., Proc. Natl. Acad. Sci. USA 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., (2003) Nat. Med. 9:129-134.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible mutations, e.g., naturally occurring mutations, that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. Monoclonal antibodies are highly specific, being directed against a single antigen. In certain embodiments, a monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody,

etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins.

The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler and Milstein, *Nature,* 256:495-97 (1975); Hongo et al., *Hybridoma,* 14 (3): 253-260 (1995), Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567), phage-display technologies (see, e.g., Clackson et al., *Nature,* 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1991); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284(1-2): 119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, e.g., WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits et al., *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits et al., *Nature* 362: 255-258 (1993); Bruggemann et al., *Year in Immunol.* 7:33 (1993); U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild et al., *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also, e.g., Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994); and U.S. Pat. Nos. 6,982,321 and 7,087,409. See also van Dijk and van de Winkel, *Curr. Opin. Pharmacol.,* 5: 368-74 (2001). Human antibodies can be prepared by administering the antigen to a transgenic animal that has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, e.g., immunized xenomice (see, e.g., U.S. Pat. Nos. 6,075,181 and 6,150,584 regarding XENOMOUSE™ technology). See also, for example, Li et al., *Proc. Natl. Acad. Sci. USA,* 103:3557-3562 (2006) regarding human antibodies generated via a human B-cell hybridoma technology. The humanized antibody may also include a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies (Vaughan et al. *Nature Biotechnology* 14:309-314 (1996): Sheets et al. *PNAS* (*USA*) 95:6157-6162 (1998)); Hoogenboom and Winter, *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581 (1991)). Human antibodies can also be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild et al., *Nature Biotechnology* 14: 845-51 (1996); Neuberger, *Nature Biotechnology* 14: 826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995). Alternatively, the human antibody may be prepared via immortalization of human B lymphocytes producing an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or may have been immunized in vitro). See, e.g., Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner et al., *J. Immunol.,* 147 (1):86-95 (1991); and U.S. Pat. No. 5,750,373.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs/HVRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al., Bio/Technology 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR/HVR and/or framework residues is described by: Barbas et al., Proc Nat. Acad. Sci. USA 91:3809-3813 (1994); Schier et al., Gene 169:147-155 (1995); Yelton et al., J. Immunol. 155:1994-2004 (1995); Jackson et al., J. Immunol. 154(7):3310-9 (1995); and Hawkins et al., J. Mol. Biol. 226:889-896 (1992).

The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "VH." The variable domain of the light chain may be referred to as "VL." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions (HVRs) both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a beta-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., Sequences *of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "hypervariable region," "HVR," or "HV," when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. For example, the term hypervariable region refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). In native antibodies, H3 and L3 display the most diversity of the six HVRs, and H3 in particular is believed to play a unique role in conferring fine specificity to antibodies. See, e.g., Xu et al., *Immunity* 13:37-45 (2000); Johnson and Wu, in *Methods in Molecular Biology* 248:1-25 (Lo, ed., Human Press, Totowa, N.J., 2003). Indeed, naturally occurring camelid antibodies consisting of a heavy chain only are functional and stable in the absence of light chain. See, e.g., Hamers-Casterman et al., *Nature* 363:446-448 (1993); Sheriff et al., *Nature Struct. Biol.* 3:733-736 (1996).

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ϵ, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas et al., Cellular and Mol. Immunology, 4th ed. (2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

The term "Fc region" is used to define the C-terminal region of an immunoglobulin heavy chain which may be generated by papain digestion of an intact antibody. The Fc region may be a native sequence Fc region or a variant Fc region. The Fc region of an immunoglobulin generally comprises two constant domains, a CH2 domain and a CH3 domain, and optionally comprises a CH4 domain.

By "Fc region chain" herein is meant one of the two polypeptide chains of an Fc region.

The "CH2 domain" of a human IgG Fc region (also referred to as "Cg2" domain) is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. It has been speculated that the carbohydrate may provide a substitute for the domain-domain pairing and help stabilize the CH2 domain. Burton, *Molec. Immunol.* 22:161-206 (1985). The CH2 domain herein may be a native sequence CH2 domain or variant CH2 domain.

The "CH3 domain" comprises the stretch of residues C-terminal to a CH2 domain in an Fc region. The CH3 region herein may be a native sequence CH3 domain or a variant CH3 domain (e.g. a CH3 domain with an introduced "protroberance" in one chain thereof and a corresponding introduced "cavity" in the other chain thereof; see U.S. Pat. No. 5,821,333, expressly incorporated herein by reference). Such variant CH3 domains may be used to make multispecific (e.g. bispecific) antibodies as herein described.

"Hinge region" herein may be a native sequence hinge region or a variant hinge region. The two polypeptide chains of a variant hinge region generally retain at least one cysteine residue per polypeptide chain, so that the two polypeptide chains of the variant hinge region can form a disulfide bond between the two chains. The preferred hinge region herein is a native sequence human hinge region, e.g. a native sequence human IgG1 hinge region.

A "functional Fc region" possesses at least one "effector function" of a native sequence Fc region. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g. an antibody variable domain) and can be assessed using various assays known in the art for evaluating such antibody effector functions.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgG1 Fc region (non-A and A allotypes); native sequence human IgG2 Fc region; native

sequence human IgG3 Fc region; and native sequence human IgG4 Fc region as well as naturally occurring variants thereof.

An "intact" antibody is one which comprises an antigen-binding variable region as well as a light chain constant domain (C_L) and heavy chain constant domains, C_H1, C_H2 and C_H3. The constant domains may be native sequence constant domains (e.g. human native sequence constant domains) or amino acid sequence variant thereof. Preferably, the intact antibody has one or more effector functions.

A "parent antibody" or "wild-type" antibody is an antibody comprising an amino acid sequence which lacks one or more amino acid sequence alterations compared to an antibody variant as herein disclosed. Thus, the parent antibody generally has at least one hypervariable region which differs in amino acid sequence from the amino acid sequence of the corresponding hypervariable region of an antibody variant as herein disclosed. The parent polypeptide may comprise a native sequence (i.e. a naturally occurring) antibody (including a naturally occurring allelic variant), or an antibody with pre-existing amino acid sequence modifications (such as insertions, deletions and/or other alterations) of a naturally occurring sequence. Throughout the disclosure, "wild type," "WT," "wt," and "parent" or "parental" antibody are used interchangeably.

As used herein, "antibody variant" or "variant antibody" refers to an antibody which has an amino acid sequence which differs from the amino acid sequence of a parent antibody. Preferably, the antibody variant comprises a heavy chain variable domain or a light chain variable domain having an amino acid sequence which is not found in nature. Such variants necessarily have less than 100% sequence identity or similarity with the parent antibody. In a preferred embodiment, the antibody variant will have an amino acid sequence from about 75% to less than 100% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the parent antibody, more preferably from about 80% to less than 100%, more preferably from about 85% to less than 100%, more preferably from about 90% to less than 100%, and most preferably from about 95% to less than 100%. The antibody variant is generally one which comprises one or more amino acid alterations in or adjacent to one or more hypervariable regions thereof.

A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification. In certain embodiments, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will typically possess, e.g., at least about 80% sequence identity with a native sequence Fc region and/or with an Fc region of a parent polypeptide, or at least about 90% sequence identity therewith, or at least about 95% sequence or more identity therewith.

Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. No. 5,500,362 or U.S. Pat. No. 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. *PNAS* (*USA*) 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. In certain embodiments, the cells express at least FcγRIII and perform ADCC effector function(s). Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being generally preferred. The effector cells may be isolated from a native source thereof, e.g. from blood or PBMCs as described herein.

"Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. In some embodiments, an FcR is a native human FcR. In some embodiments, an FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of those receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see, e.g., Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed, for example, in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein.

The term "Fc receptor" or "FcR" also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)) and regulation of homeostasis of immunoglobulins. Methods of measuring binding to FcRn are known (see, e.g., Ghetie and Ward., *Immunol. Today* 18(12):592-598 (1997); Ghetie et al., *Nature Biotechnology,* 15(7):637-640 (1997); Hinton et al., *J. Biol. Chem.* 279(8):6213-6216 (2004); WO 2004/92219 (Hinton et al.).

Binding to human FcRn in vivo and serum half life of human FcRn high affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates to which the

polypeptides with a variant Fc region are administered. WO 2000/42072 (Presta) describes antibody variants with improved or diminished binding to FcRs. See also, e.g., Shields et al. *J. Biol. Chem.* 9(2):6591-6604 (2001).

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (Clq) to antibodies (of the appropriate subclass), which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g., as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed. Polypeptide variants with altered Fc region amino acid sequences (polypeptides with a variant Fc region) and increased or decreased C1q binding capability are described, e.g., in U.S. Pat. No. 6,194,551 B1 and WO 1999/51642. See also, e.g., Idusogie et al. *J. Immunol.* 164: 4178-4184 (2000).

An "affinity matured" antibody is one with one or more alterations in one or more CDRs thereof which result an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). In one embodiment, an affinity matured antibody has nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. *Bio/Technology* 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al. *Proc Nat. Acad. Sci, USA* 91:3809-3813 (1994); Schier et al. *Gene* 169:147-155 (1995); Yelton et al. *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al, *J. Mol. Biol.* 226:889-896 (1992).

The term "therapeutic antibody" refers to an antibody that is used in the treatment of disease. A therapeutic antibody may have various mechanisms of action. A therapeutic antibody may bind and neutralize the normal function of a target associated with an antigen. For example, a monoclonal antibody that blocks the activity of the of protein needed for the survival of a cancer cell causes the cell's death. Another therapeutic monoclonal antibody may bind and activate the normal function of a target associated with an antigen. For example, a monoclonal antibody can bind to a protein on a cell and trigger an apoptosis signal. Yet another monoclonal antibody may bind to a target antigen expressed only on diseased tissue; conjugation of a toxic payload (effective agent), such as a chemotherapeutic or radioactive agent, to the monoclonal antibody can create an agent for specific delivery of the toxic payload to the diseased tissue, reducing harm to healthy tissue. A "biologically functional fragment" of a therapeutic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

The antibody may bind to any protein, including, without limitation, a member of the HER receptor family, such as HER1 (EGFR), HER2, HER3 and HER4; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD21, CD22, and CD34; cell adhesion molecules such as LFA-1, Mol, p150,95, VLA-4, ICAM-1, VCAM and av/p3 integrin including either α or β or subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as vascular endothelial growth factor (VEGF); IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; and protein C. Other exemplary proteins include growth hormone (GH), including human growth hormone (hGH) and bovine growth hormone (bGH); growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA); bombazine; thrombin; tumor necrosis factor-α and -β; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); serum albumin such as human serum albumin (HSA); mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins (IGFBPs); erythropoietin (EPO); thrombopoietin (TPO); osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and biologically active fragments or variants of any of the above-listed polypeptides. Many other antibodies and/or other proteins may be used in accordance with the instant invention, and the above lists are not meant to be limiting.

Therapeutic antibodies of particular interest include those in clinical oncological practice or development such as commercially available AVASTIN® (bevacizumab), HERCEPTIN® (trastuzumab), LUCENTIS® (ranibizumab), RAPTIVA® (efalizumab), RITUXAN® (rituximab), and XOLAIR® (omalizumab), as well as, anti-amyloid beta (Abeta), anti-CD4 (MTRX1011A), anti-EGFL7 (EGF-like-domain 7), anti-IL13, Apomab (anti-DR5-targeted pro-apoptotic receptor agonist (PARA), anti-BR3 (CD268, BLyS receptor 3, BAFF-R, BAFF Receptor), anti-beta 7 integrin subunit, dacetuzumab (Anti-CD40), GA101 (anti-CD20 monoclonal antibody), MetMAb (anti-MET receptor tyrosine kinase), anti-neuropilin-1 (NRP1), ocrelizumab (anti-CD20 antibody), anti-OX40 ligand, anti-oxidized LDL (oxLDL), pertuzumab (HER dimerization inhibitors (HDIs), and. rhuMAb IFN alpha.

A "biologically functional fragment" of an antibody comprises only a portion of an intact antibody, wherein the portion retains at least one, and as many as most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, a biologically functional fragment of an antibody comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, a biologically functional fragment of an antibody, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, a

biologically functional fragment of an antibody is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such a biologically functional fragment of an antibody may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

The term "diagnostic protein" refers to a protein that is used in the diagnosis of a disease.

The term "diagnostic antibody" refers to an antibody that is used as a diagnostic reagent for a disease. The diagnostic antibody may bind to a target antigen that is specifically associated with, or shows increased expression in, a particular disease. The diagnostic antibody may be used, for example, to detect a target in a biological sample from a patient, or in diagnostic imaging of disease sites, such as tumors, in a patient. A "biologically functional fragment" of a diagnostic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

"Purified" means that a molecule is present in a sample at a concentration of at least 80-90% by weight of the sample in which it is contained. The protein, including antibodies, which is purified is preferably essentially pure and desirably essentially homogeneous (i.e. free from contaminating proteins etc.).

An "essentially pure" protein means a protein composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight.

An "essentially homogeneous" protein means a protein composition comprising at least about 99% by weight of protein, based on total weight of the composition.

As used herein, "soluble" refers to polypeptides that, when in aqueous solutions, are completely dissolved, resulting in a clear to slightly opalescent solution with no visible particulates, as assessed by visual inspection. A further assay of the turbidity of the solution (or solubility of the protein) may be made by measuring UV absorbances at 340 nm to 360 nm with a 1 cm path-length cell where turbidity at 20 mg/ml is less than 0.05 absorbance units.

An "isolated" antibody or polypeptide is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater than 99% by weight; (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The terms "Protein A" and "ProA" are used interchangeably herein and encompasses Protein A recovered from a native source thereof, Protein A produced synthetically (e.g. by peptide synthesis or by recombinant techniques), and variants thereof which retain the ability to bind proteins which have a C_H2/C_H3 region, such as an Fc region. Protein A can be purchased commercially from Repligen, Pharmacia and Fermatech. Protein A is generally immobilized on a solid phase support material. The term "ProA" also refers to an affinity chromatography resin or column containing chromatographic solid support matrix to which is covalently attached Protein A.

The term "chromatography" refers to the process by which a solute of interest in a mixture is separated from other solutes in a mixture as a result of differences in rates at which the individual solutes of the mixture migrate through a stationary medium under the influence of a moving phase, or in bind and elute processes.

The term "affinity chromatography" and "protein affinity chromatography" are used interchangeably herein and refer to a protein separation technique in which a protein of interest or antibody of interest is reversibly and specifically bound to a biospecific ligand. Preferably, the biospecific ligand is covalently attached to a chromatographic solid phase material and is accessible to the protein of interest in solution as the solution contacts the chromatographic solid phase material. The protein of interest (e.g., antibody, enzyme, or receptor protein) retains its specific binding affinity for the biospecific ligand (antigen, substrate, cofactor, or hormone, for example) during the chromatographic steps, while other solutes and/or proteins in the mixture do not bind appreciably or specifically to the ligand. Binding of the protein of interest to the immobilized ligand allows contaminating proteins or protein impurities to be passed through the chromatographic medium while the protein of interest remains specifically bound to the immobilized ligand on the solid phase material. The specifically bound protein of interest is then removed in active form from the immobilized ligand with low pH, high pH, high salt, competing ligand, and the like, and passed through the chromatographic column with the elution buffer, free of the contaminating proteins or protein impurities that were earlier allowed to pass through the column. Any component can be used as a ligand for purifying its respective specific binding protein, e.g. antibody.

The terms "non-affinity chromatography" and "non-affinity purification" refer to a purification process in which affinity chromatography is not utilized. Non-affinity chromatography includes chromatographic techniques that rely on non-specific interactions between a molecule of interest (such as a protein, e.g. antibody) and a solid phase matrix.

A "cation exchange resin" refers to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin may, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-methyl-cellulose, sulphopropyl (SP) immobilized on agarose (e.g. SP-SEPHAROSE FAST FLOW™ or SP-SEPHAROSE HIGH PERFORMANCE™, from Pharmacia) and sulphonyl immobilized on agarose (e.g. S-SEPHAROSE FAST FLOW™ from Pharmacia). A "mixed mode ion exchange resin" refers to a solid phase which is covalently modified with cationic, anionic, and hydrophobic moieties. A commercially available mixed mode ion exchange resin is BAKERBOND ABX™ (J.T. Baker, Phillipsburg, N.J.) containing weak cation exchange groups, a low concentration of anion exchange groups, and hydrophobic ligands attached to a silica gel solid phase support matrix.

The term "anion exchange resin" is used herein to refer to a solid phase which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Commercially available anion exchange resins include DEAE cellulose, QAE SEPHADEX™ and FAST Q SEPHAROSE™ (Pharmacia).

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in *Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., ed. Calbiochem Corporation (1975). In one embodiment, the buffer has a pH in the range from about 2 to about 9, alternatively from about 3 to about 8, alternatively from about 4 to about 7 alternatively from about 5 to about 7. Non-limiting examples of buffers that will control the pH in this range include MES, MOPS, MOPSO, Tris, HEPES, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

The "loading buffer" is that which is used to load the composition comprising the polypeptide molecule of interest and one or more impurities onto the ion exchange resin. The loading buffer has a conductivity and/or pH such that the polypeptide molecule of interest (and generally one or more impurities) is/are bound to the ion exchange resin or such that the protein of interest flows through the column while the impurities bind to the resin.

The "intermediate buffer" is used to elute one or more impurities from the ion exchange resin, prior to eluting the polypeptide molecule of interest. The conductivity and/or pH of the intermediate buffer is/are such that one or more impurity is eluted from the ion exchange resin, but not significant amounts of the polypeptide of interest.

The term "wash buffer" when used herein refers to a buffer used to wash or re-equilibrate the ion exchange resin, prior to eluting the polypeptide molecule of interest. Conveniently, the wash buffer and loading buffer may be the same, but this is not required.

The "elution buffer" is used to elute the polypeptide of interest from the solid phase. The conductivity and/or pH of the elution buffer is/are such that the polypeptide of interest is eluted from the ion exchange resin.

A "regeneration buffer" may be used to regenerate the ion exchange resin such that it can be re-used. The regeneration buffer has a conductivity and/or pH as required to remove substantially all impurities and the polypeptide of interest from the ion exchange resin.

The term "substantially similar" or "substantially the same," as used herein, denotes a sufficiently high degree of similarity between two numeric values (for example, one associated with an antibody of the invention and the other associated with a reference/comparator antibody), such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, and/or less than about 10% as a function of the reference/comparator value.

The phrase "substantially reduced," or "substantially different," as used herein with regard to amounts or numerical values (and not as reference to the chemical process of reduction), denotes a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, and/or greater than about 50% as a function of the value for the reference/comparator molecule.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors," or simply, "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector.

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, Calif., or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B.

It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

"Percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a reference Factor D-encoding sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. Sequence identity is then calculated relative to the longer sequence, i.e. even if a shorter sequence shows 100% sequence identity with a portion of a longer sequence, the overall sequence identity will be less than 100%.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. "Treatment" herein encompasses alleviation of the disease and of the signs and symptoms of the particular disease.

A "disorder" is any condition that would benefit from treatment with the protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include carcinomas and allergies.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, non-human higher primates, other vertebrates, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

B. Exemplary Methods and Materials for Carrying Out the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology and the like, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., *Molecular Cloning: A Laboratory Manual*, (J. Sambrook et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989); *Current Protocols in Molecular Biology* (F. Ausubel et al., eds., 1987 updated); *Essential Molecular Biology* (T. Brown ed., IRL Press 1991); *Gene Expression Technology* (Goeddel ed., Academic Press 1991); *Methods for Cloning and Analysis of Eukaryotic Genes* (A. Bothwell et al., eds., Bartlett Publ. 1990); *Gene Transfer and Expression* (M. Kriegler, Stockton Press 1990); *Recombinant DNA Methodology II* (R. Wu et al., eds., Academic Press 1995); PCR: *A Practical Approach* (M. McPherson et al., IRL Press at Oxford University Press 1991); *Oligonucleotide Synthesis* (M. Gait ed., 1984); *Cell Culture for Biochemists* (R. Adams ed., Elsevier Science Publishers 1990); *Gene Transfer Vectors for Mammalian Cells* (J. Miller & M. Calos eds., 1987); *Mammalian Cell Biotechnology* (M. Butler ed., 1991); *Animal Cell Culture* (J. Pollard et al., eds., Humana Press 1990); *Culture of Animal Cells*, 2nd Ed. (R. Freshney et al., eds., Alan R. Liss 1987); *Flow Cytometry and Sorting* (M. Melamed et al., eds., Wiley-Liss 1990); the series *Methods in Enzymology* (Academic Press, Inc.); Wirth M. and Hauser H. (1993); *Immunochemistry in Practice*, 3rd edition, A. Johnstone & R. Thorpe, Blackwell Science, Cambridge, Mass., 1996; *Techniques in Immunocytochemistry*, (G. Bullock & P. Petrusz eds., Academic Press 1982, 1983, 1985, 1989); Handbook of Experimental Immunology, (D. Weir & C. Blackwell, eds.); *Current Protocols in Immunology* (J. Coligan et al., eds. 1991); *Immunoassay* (E. P. Diamandis & T. K. Christopoulos, eds., Academic Press, Inc., 1996); Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed) Academic Press, New York; Ed Harlow and David Lane, *Antibodies A laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988; *Antibody Engineering*, 2nd edition (C. Borrebaeck, ed., Oxford University Press, 1995); and the series *Annual Review of Immunology*; the series *Advances in Immunology*.

1. Recombinant Production of Proteins in Mammalian Host Cells Using a Glutamine-Free Cell Culture Medium

The present invention concerns the large-scale recombinant production of proteins in mammalian host cells, using a glutamine-free cell culture medium supplemented with asparagine. Mammalian cells have become the dominant system for the production of mammalian proteins for clinical applications, primarily due to their ability to produce properly folded and assembled heterologous proteins, and their capacity for post-translational modifications. Chinese hamster ovary (CHO) cells, and cell lines obtained from various other mammalian sources, such as, for example, mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) and human retinal cells have been approved by regulatory agencies for the production of biopharmaceutical products, including therapeutic antibodies. Of these, Chinese Hamster Ovary Cells (CHO) are among the most commonly used industrial hosts, which are widely employed for the production of heterologous proteins. Thus, methods for the large-scale production of antibodies in CHO, including dihydrofolate reductase negative (DHFR−) CHO cells, are well known in the art (see, e.g. Trill et al., *Curr. Opin. Biotechnol.* 6(5):553-60 (1995) and U.S. Pat. No. 6,610,516).

As a first step, the nucleic acid (e.g., cDNA or genomic DNA) encoding the desired recombinant protein may be inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below. Optional signal sequences, origins of replication, marker genes, enhancer elements and transcription terminator sequences that may be employed are known in the art and described in further detail in PCT Publication WO 97/25428.

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the protein-encoding nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the

presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding the desired protein by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector.

Promoters suitable for use with prokaryotic and eukaryotic hosts are known in the art, and are described in further detail in PCT Publication No. WO97/25428.

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* cells, such as *E. coli* K12 strain 294 (ATCC® 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced using standard techniques known in the art. (See, e.g., Messing et al., *Nucleic Acids Res.* 1981, 9:309; Maxam et al., Methods in Enzymology 1980, 65:499).

Expression vectors that provide for the transient expression in mammalian cells may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector (Sambrook et al., supra). Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of a desired heterologous protein in recombinant vertebrate cell culture are described in Gething et al., *Nature* 1981, 293:620-625; Mantei et al., *Nature* 1979, 281:40-46; EP 117,060; and EP 117,058.

For large-scale production, according to the present invention mammalian host cells are transfected and preferably transformed with the above-described expression vectors and cultured in nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with Agrobacterium tumefaciens is used for transformation of certain plant cells, as described (Shaw et al., Gene 1983, 23:315 and PCT Publication No. WO 89/05859). In addition, plants may be transfected using ultrasound treatment, PCT Publication No. WO 91/00358 published 10 Jan. 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method (Graham and van der Eb, *Virology* 1978, 52:456-457) may be employed. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. For various techniques for transforming mammalian cells, see also Keown et al. *Methods in Enzymology* 1990, 185:527-537 and Mansour et al. *Nature* 1988, 336:348-352.

During large-scale production, to begin the production cycle usually a small number of transformed recombinant host cells is allowed to grow in culture for several days. Once the cells have undergone several rounds of replication, they are transferred to a larger container where they are prepared to undergo fermentation. The media in which the cells are grown and the levels of oxygen, nitrogen and carbon dioxide that exist during the production cycle may have a significant impact on the production process. Growth parameters are determined specifically for each cell line and these parameters are measured frequently to assure optimal growth and production conditions.

When the cells grow to sufficient numbers, they are transferred to large-scale production tanks to begin the production phase, and grown for a longer period of time. At this point in the process, the recombinant protein can be harvested. Typically, the cells are engineered to secrete the polypeptide into the cell culture media, so the first step in the purification process is to separate the cells from the media. Harvesting usually includes centrifugation and filtration to produce a Harvested Cell Culture Fluid (HCCF). The media is then subjected to several additional purification steps that remove any cellular debris, unwanted proteins, salts, minerals or other undesirable elements. At the end of the purification process, the recombinant protein is highly pure and is suitable for human therapeutic use.

Although this process has been the subject of much study and improvements over the past several decades, there is room fur further improvements in the large-scale commercial production of recombinant proteins, such as antibodies. Thus, increases in cell viability, longevity and specific productivity of mammalian host cell cultures, and improvements in the titer of the recombinant proteins produced have a genuine impact on the price of the recombinant protein produced, and, in the case of therapeutic proteins, the price and availability of drug products.

The present invention concerns an improved method for the production of heterologous proteins in mammalian cell culture, using a glutamine-free culture medium with added asparagine in the production phase of the cell culture process. The culture media used in the process of the present invention can be based on any commercially available medium for recombinant production of proteins in mammalian host cells, in particular CHO cells.

Examples of commercially available culture media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be

included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. In addition, the culture media of the present invention can be based any of the media described in Ham and McKeehan, Meth. Enz., 58: 44 (1979); Barnes and Sato, Anal. Biochem., 102: 255 (1980); U.S. Pat. No. 4,767,704; U.S. Pat. No. 4,657,866; U.S. Pat. No. 4,927,762; U.S. Pat. No. 5,122,469 or U.S. Pat. No. 4,560,655; WO 90/03430; and WO 87/00195, provided that glutamine is omitted as an ingredient.

Under Glutamine-free conditions Asparagine is required since mammalian cells can synthesize Asparagine only in presence of Glutamine. Asparagine is synthesized by amide transfer from Glutamine in the presence of Asparagine synthetase. The Asparagine is preferably added to the culture medium at a concentration in the range of 2.5 mM to 15 mM. In various embodiments of the present invention, the preferred concentration of Asparagine should be at least 2.5 mM. In preferred embodiments, the asparagine is added at a concentration of 10 mM.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in, and can be adapted for the production of recombinant proteins using the cell culture media herein.

The necessary nutrients and growth factors for the medium, including their concentrations, for a particular cell line, are determined empirically without undue experimentation as described, for example, in *Mammalian Cell Culture*, Mather, ed. (Plenum Press: NY, 1984); Barnes and Sato, *Cell,* 22: 649 (1980) or *Mammalian Cell Biotechnology: A Practical Approach*, M. Butler, ed. (IRL Press, 1991). A suitable medium contains a basal medium component such as a DMEM/HAM F-12-based formulation (for composition of DMEM and HAM F12 media and especially serum-free media, see culture media formulations in American Type Culture Collection Catalogue of Cell Lines and Hybridomas, Sixth Edition, 1988, pages 346-349), with modified concentrations of some components such as amino acids, salts, sugar, and vitamins, and optionally containing glycine, hypoxanthine, and thymidine; recombinant human insulin, hydrolyzed peptone, such as PRIMATONE HS™ or PRIMATONE RL™ (Sheffield, England), or the equivalent; a cell protective agent, such as PLURONIC F68™ or the equivalent pluronic polyol; GENTAMYCIN™; and trace elements. The formulations of medium as described in U.S. Pat. No. 5,122,469, characterized by the presence of high levels of certain amino acids, as well as PS-20 as described below, are particularly appropriate.

The glycoproteins of the present invention may be produced by growing cells which express the desired glycoprotein under a variety of cell culture conditions. For instance, cell culture procedures for the large- or small-scale production of glycoproteins are potentially useful within the context of the present invention. Procedures including, but not limited to, a fluidized bed bioreactor, hollow fiber bioreactor, roller bottle culture, or stirred tank bioreactor system may be used, in the later two systems, with or without microcarriers, and operated alternatively in a batch, fed-batch, or continuous mode.

In a particular embodiment the cell culture of the present invention is performed in a stirred tank bioreactor system and a fed-batch culture procedure is employed. In the preferred fed-batch culture the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed-batch culture can include, for example, a semi-continuous fed-batch culture, wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium Fed-batch culture is distinguished from simple-batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed-batch culture can be further distinguished from perfusion culturing insofar as the supernate is not removed from the culturing vessel during the process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers, etc., and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cells of the culture may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, the present invention contemplates a single-step or multiple-step culture procedure. In a single-step culture the host cells are inoculated into a culture environment and the processes of the instant invention are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture is envisioned. In the multi-stage culture cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

According to a specific aspect of the invention, fed-batch or continuous cell culture conditions are devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In the growth phase cells are grown under conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (DO_2), and the like, are those used with the particular host and will be apparent to the ordinarily-skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO_2) or a base (e.g., Na_2CO_3 or NaOH). A suitable temperature range for culturing mammalian cells such as CHO cells is between about 30 to 40° C. and preferably about 37° C. and a suitable DO_2 is between 5-90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above the production phase or step may be continuous with the inoculation or growth phase or step.

Production of a target protein in mammalian, e.g., CHO, cells typically employs a semi-continuous process whereby cells are culture in a "seed-train" for various periods of time and are periodically transferred to inoculum fermentors to generate enough cell mass to inoculate a production fermentor at larger scale. Thus, cells used for the production of the desired protein are in culture for various periods of time up to a maximum predefined cell age. The parameters of the cell culture process, such as seed density, pH, DO_2 and temperature during culture, duration of the production culture, operating conditions of harvest, etc. are a function of the particular cell line and culture medium used, and can be determined empirically, without undue experimentation.

According to the present invention, the cell-culture environment during the production phase of the cell culture is controlled. In a preferred aspect, the production phase of the

cell culture process is preceded by a transition phase of the cell culture in which parameters for the production phase of the cell culture are engaged.

The desired polypeptide, such as antibody, preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly produced without a secretory signal. If the polypeptide is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g., Triton-X 100) or its extracellular region may be released by enzymatic cleavage.

When the polypeptide is produced in a recombinant cell other than one of human origin, it is free of proteins or polypeptides of human origin. However, it is usually necessary to recover or purify recombinant proteins from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the desired polypeptide. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. The heterologous polypeptide thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column such as SP-Sepharose™ or CM-Sepharose™; hydroxyapatite; hydrophobic interaction chromatography; ethanol precipitation;

chromatofocusing; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75™; and/or diafiltration.

Recombinant polypeptides can be isolated, e.g. by affinity chromatography.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for the purification and isolation of recombinant proteins, including antibodies, can be used herein, and modified if needed, using standard techniques.

Expression of the desired heterologous protein may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA* 1980, 77:5201-5205), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal

2. Antibodies

In a preferred embodiment, the methods of the present invention are used for the recombinant production of antibodies, including therapeutic and diagnostic antibodies. Antibodies within the scope of the present invention include, but are not limited to: anti-HER2 antibodies including Trastuzumab (HERCEPTIN®) (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285-4289 (1992), U.S. Pat. No. 5,725,856); anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN®), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108B1, or Tositumomab (BEXXAR®); anti-IL-8 (St John et al., *Chest*, 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors*, 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., *Transplant Intl.* 4:3-7 (1991), and Hourmant et al., *Transplantation* 58:377-380 (1994)); anti-IgE (Presta et al., *J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul. 31, 1997); anti-IgE (including E25, E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No. 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No. PCT/US98/13410 filed Jun. 30, 1998, U.S. Pat. No. 5,714,338); anti-Apo-2 receptor antibody (WO 98/51793 published Nov. 19, 1998); anti-TNF-α antibodies including cA2 (REMICADE®), CDP571 and MAK-195 (See, U.S. Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al., *J. Immunol.* 156(4):1646-1653 (1996), and Dhainaut et al., *Crit. Care Med.* 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human $\alpha_4\beta_7$ integrin (WO 98/06248 published Feb. 19, 1998); anti-EGFR (chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S. Pat. No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-tac antibodies such as CHI-621 (SIMULECT®) and (ZENAPAX®) (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997); anti-CD4 antibodies such as the cM-7412 antibody (Choy et al., *Arthritis Rheum* 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al., *Nature* 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22 antibody directed against FcγRI as in Graziano et al., *J. Immunol.* 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al., *Cancer Res.* 55(23Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al., *Cancer Res.* 55(23): 5852s-5856s (1995); and Richman et al., *Cancer Res.* 55(23 Supp): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al., *Eur J. Immunol.* 26(1):1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al., *J. Immunol.* 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al., *Cancer Res* 55(23 Suppl):5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22 antibodies such as LL2 or Lympho-

Cide (Juweid et al., *Cancer Res* 55(23 Suppl):5899s-5907s (1995)); anti-EpCAM antibodies such as 17-1A (PANOREX®); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO®); anti-RSV antibodies such as MEDI-493 (SYNAGIS®); anti-CMV antibodies such as PROTOVIR®; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR®; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-αvβ3 antibody VITAXIN®; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1). The preferred target antigens for the antibody herein are: HER2 receptor, VEGF, IgE, CD20, CD11a, and CD40.

Many of these antibodies are widely used in clinical practice to treat various diseases, including cancer.

In certain specific embodiments, the methods of the present invention are used for the production of the following antibodies and recombinant proteins.

Anti-CD20 Antibodies

Rituximab (RITUXAN®) is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. Rituximab is the antibody called "C2B8" in U.S. Pat. No. 5,736,137 issued Apr. 7, 1998 (Anderson et al.). Rituximab is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20-positive, B cell non-Hodgkin's lymphoma. In vitro mechanism of action studies have demonstrated that rituximab binds human complement and lyses lymphoid B cell lines through complement-dependent cytotoxicity (CDC) (Reff et al., *Blood* 83(2): 435-445 (1994)). Additionally, it has significant activity in assays for antibody-dependent cellular cytotoxicity (ADCC). More recently, rituximab has been shown to have anti-proliferative effects in tritiated thymidine incorporation assays and to induce apoptosis directly, while other anti-CD19 and CD20 antibodies do not (Maloney et al., *Blood* 88(10):637a (1996)). Synergy between rituximab and chemotherapies and toxins has also been observed experimentally. In particular, rituximab. sensitizes drug-resistant human B cell lymphoma cell lines to the cytotoxic effects of doxorubicin, CDDP, VP-1 6, diphtheria toxin and ricin (Demidem et al., *Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997)). In vivo preclinical studies have shown that rituximab depletes B cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff et al., *Blood* 83(2): 435-445 (1994)).

Patents and patent publications concerning CD20 antibodies include U.S. Pat. Nos. 5,776,456, 5,736,137, 6,399,061, and 5,843,439, as well as U.S. patent application Nos. US 2002/0197255A1, US 2003/0021781A1, US 2003/0082172 A1, US 2003/0095963 A1, US 2003/0147885 A1 (Anderson et al.); U.S. Pat. No. 6,455,043B1 and WO00/09160 (Grillo-Lopez, A.); WO00/27428 (Grillo-Lopez and White); WO00/27433 (Grillo-Lopez and Leonard); WO00/44788 (Braslawsky et al.); WO01/10462 (Rastetter, W.); WO01/10461 (Rastetter and White); WO01/10460 (White and Grillo-Lopez); U.S. application No. US2002/0006404 and WO02/04021 (Hanna and Hariharan); U.S. application No. US2002/0012665 A1 and WO01/74388 (Hanna, N.); U.S. application No. US 2002/0058029 A1 (Hanna, N.); U.S. application No. US 2003/0103971 A1 (Hariharan and Hanna); U.S. application No. US2002/0009444A1, and WO01/80884 (Grillo-Lopez, A.); WO01/97858 (White, C.); U.S. application No. US2002/0128488A1 and WO02/34790 (Reff, M.); W)02/060955 (Braslawsky et al.); WO2/096948 (Braslawsky et al.); WO02/079255 (Reff and Davies); U.S. Pat. No. 6,171,586B1, and WO98/56418 (Lam et al.); WO98/58964 (Raju, S.); WO99/22764 (Raju, S.); WO99/51642, U.S. Pat. No. 6,194,551B1, U.S. Pat. No. 6,242,195B1, U.S. Pat. No. 6,528,624B1 and U.S. Pat. No. 6,538,124 (Idusogie et al.); WO00/42072 (Presta, L.); WO00/67796 (Curd et al.); WO01/03734 (Grillo-Lopez et al.); U.S. application No. US 2002/0004587A1 and WO01/77342 (Miller and Presta); U.S. application No. US2002/0197256 (Grewal, I.); U.S. application No. US 2003/0157108 A1 (Presta, L.); U.S. Pat. Nos. 6,090,365B1, 6,287,537B1, 6,015,542, 5,843,398, and 5,595,721, (Kaminski et al.); U.S. Pat. Nos. 5,500,362, 5,677,180, 5,721,108, and 6,120,767 (Robinson et al.); U.S. Pat. No. 6,410,391B1 (Raubitschek et al.); U.S. Pat. No. 6,224,866B1 and WO00/20864 (Barbera-Guillem, E.); WO01/13945 (Barbera-Guillem, E.); WO00/67795 (Goldenberg); U.S. application No. US 2003/01339301 A1 and WO00/74718 (Goldenberg and Hansen); WO00/76542 (Golay et al.); WO01/72333 (Wolin and Rosenblatt); U.S. Pat. No. 6,368,596B1 (Ghetie et al.); U.S. application No. US2002/0041847 A1, (Goldenberg, D.); U.S. application No. US2003/0026801A1 (Weiner and Hartmann); WO02/102312 (Engleman, E.); U.S. patent application No. 2003/0068664 (Albitar et al.); WO03/002607 (Leung, S.); WO 03/049694 and US 2003/0185796 A1 (Wolin et al.); WO03/061694 (Sing and Siegall); US 2003/0219818 A1 (Bohen et al.); US 2003/0219433 A1 and WO 03/068821 (Hansen et al.) each of which is expressly incorporated herein by reference. See, also, U.S. Pat. No. 5,849,898 and EP application no. 330,191 (Seed et al.); U.S. Pat. No. 4,861,579 and EP332,865A2 (Meyer and Weiss); U.S. Pat. No. 4,861,579 (Meyer et al.) and WO95/03770 (Bhat et al.).

Publications concerning therapy with Rituximab include: Perotta and Abuel "Response of chronic relapsing ITP of 10 years duration to Rituximab" Abstract #3360 *Blood* 10(1) (part 1-2): p. 88B (1998); Stashi et al., "Rituximab chimeric anti-CD20 monoclonal antibody treatment for adults with chronic idopathic thrombocytopenic purpura" *Blood* 98(4): 952-957 (2001); Matthews, R. "Medical Heretics" New Scientist (7 Apr. 2001); Leandro et al., "Clinical outcome in 22 patients with rheumatoid arthritis treated with B lymphocyte depletion" *Ann Rheum Dis* 61:833-888 (2002); Leandro et al., "Lymphocyte depletion in rheumatoid arthritis: early evidence for safety, efficacy and dose response. *Arthritis & Rheumatism* 44(9): 5370 (2001); Leandro et al., "An open study of B lymphocyte depletion in systemic lupus erythematosus", *Arthritis & Rheumatism* 46(1):2673-2677 (2002); Edwards and Cambridge "Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes" *Rheumatology* 40:205-211 (2001); Edwards et al., "B-lymphocyte depletion therapy in rheumatoid arthritis and other autoimmune disorders" *Biochem. Soc. Trans.* 30(4):824-828 (2002); Edwards et al., "Efficacy and safety of Rituximab, a B-cell targeted chimeric monoclonal antibody: A randomized, placebo controlled trial in patients with rheumatoid arthritis. *Arthritis & Rheumatism* 46(9): 5197 (2002); Levine and Pestronk "IgM antibody-related polyneuropathies: B-cell depletion chemotherapy using Rituximab" *Neurology* 52: 1701-1704 (1999); DeVita et al., "Efficacy of selective B cell blockade in the treatment of rheumatoid arthritis" *Arthritis & Rheumatism* 46:2029-2033 (2002); Hidashida et al., "Treatment of DMARD-Refractory rheumatoid arthritis with rituximab." Presented at the Annual Scientific Meeting of the American College of Rheumatology;

October 24-29; New Orleans, La. 2002; Tuscano, J. "Successful treatment of Infliximab-refractory rheumatoid arthritis with rituximab" Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002. Sarwal et al., *N. Eng. J. Med.* 349(2):125-138 (Jul. 10, 2003) reports molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling.

In various embodiments, the invention provides pharmaceutical compositions comprising humanized anti-CD20 antibodies. In certain embodiments, the humanized antibody composition of the invention further comprises amino acid alterations in the IgG Fc and exhibits increased binding affinity for human FcRn over an antibody having wild-type IgG Fc, by at least 60 fold, at least 70 fold, at least 80 fold, more preferably at least 100 fold, preferably at least 125 fold, even more preferably at least 150 fold to about 170 fold.

The N-glycosylation site in IgG is at Asn297 in the C_H2 domain. Humanized antibody compositions of the present invention include compositions of any of the preceding humanized antibodies having an Fc region, wherein about 80-100% (and preferably about 90-99%) of the antibody in the composition comprises a mature core carbohydrate structure which lacks fucose, attached to the Fc region of the glycoprotein. Such compositions were demonstrated herein to exhibit a surprising improvement in binding to Fc(RIIIA (F158), which is not as effective as Fc(RIIIA (V158) in interacting with human IgG. Fc(RIIIA (F158) is more common than Fc(RIIIA (V 158) in normal, healthy African Americans and Caucasians. See Lehrnbecher et al., *Blood* 94:4220 (1999). Historically, antibodies produced in Chinese Hamster Ovary Cells (CHO), one of the most commonly used industrial hosts, contain about 2 to 6% in the population that are nonfucosylated. YB2/0 and Lec13, however, can produce antibodies with 78 to 98% nonfucosylated species. Shinkawa et al., *J Bio. Chem.* 278 (5), 3466-347 (2003), reported that antibodies produced in YB2/0 and Lec13 cells, which have less FUT8 activity, show significantly increased ADCC activity in vitro. The production of antibodies with reduced fucose content are also described in e.g., Li et al., (GlycoFi) "Optimization of humanized IgGs in glycoengineered *Pichia pastoris"* in Nature Biology online publication 22 Jan. 2006; Niwa R. et al., *Cancer Res.* 64(6):2127-2133 (2004); US 2003/0157108 (Presta); U.S. Pat. No. 6,602,684 and US 2003/0175884 (Glycart Biotechnology); US 2004/0093621, US 2004/0110704, US 2004/0132140 (all of Kyowa Hakko Kogyo).

A bispecific humanized antibody encompasses an antibody wherein one arm of the antibody has at least the antigen binding region of the H and/or L chain of a humanized antibody of the invention, and the other arm has V region binding specificity for a second antigen. In specific embodiments, the antigens are selected from the group consisting of CD-20, CD3, CD64, CD32A, CD16, NKG2D or other NK activating ligands.

Anti-HER2 Antibodies

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, trastuzumab or HERCEPTIN®; U.S. Pat. No. 5,821,337) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). Trastuzumab received marketing approval from the Food and Drug Administration (FDA) Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein. In November 2006, the FDA approved Herceptin as part of a treatment regimen containing doxorubicin, cyclophosphamide and paclitaxel, for the adjuvant treatment of patients with HER2-positive, node-positive breast cancer.

In various embodiments, the invention provides pharmaceutical compositions comprising humanized anti-HER2 antibodies. HER2 antibodies with various properties have been described in Tagliabue et al., *Int. J. Cancer* 47:933-937 (1991); McKenzie et al., *Oncogene* 4:543-548 (1989); Maier et al., *Cancer Res.* 51:5361-5369 (1991); Bacus et al., *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al., *PNAS* (*USA*) 88:8691-8695 (1991); Bacus et al., *Cancer Research* 52:2580-2589 (1992); Xu et al., *Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk et al., *Cancer Research* 52:2771-2776 (1992); Hancock et al., *Cancer Res.* 51:4575-4580 (1991); Shawver et al., *Cancer Res.* 54:1367-1373 (1994); Arteaga et al., *Cancer Res.* 54:3758-3765 (1994); Harwerth et al., *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; and Klapper et al., *Oncogene* 14:2099-2109 (1997).

Anti-VEGF Antibodies

anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors,* 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998) are FDA approved for the treatment of cancer. In various embodiments, the invention provides pharmaceutical compositions comprising humanized anti-VEGF antibodies.

Anti-CD11a Antibodies

The humanized anti-CD11a antibody efalizumab or Raptiva (U.S. Pat. No. 6,037,454) received marketing approval from the Food and Drug Administration on Oct. 27, 2003 for the treatment for the treatment of psoriasis. One embodiment provides for pharmaceutical compositions comprising anti-human CD11a antibodies.

Apomab Antibodies

Antibodies to the DR5 receptor (anti-DR5) antibodies can also be produced in accordance with the present invention. Such anti-DR5 antibodies specifically include all antibody variants disclosed in PCT Publication No. WO 2006/083971, such as the anti-DR5 antibodies designated Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3, especially Apomab 8.3 and Apomab 7.3, preferably Apomab 7.3. The entire content of WO 2006/083971 is hereby expressly incorporated by reference. Apomab is a fully human monoclonal antibody which is a DR5-targeted pro-apoptotic receptor agonist (PARA) specifically designed to induce apoptosis. Apoptosis is a natural process by which damaged or unwanted cells, including those that are cancerous, die and are cleared from the body. Pro-apoptotic receptor DR5 is expressed in a broad range of malignancies.

Anti-BR3 Antibodies and Immunoadhesins

Antibodies to the BR3 (anti-BR3) antibodies and BR3-Fc immunoadhesins can also be produced in accordance with the present invention. Such anti-BR3 antibodies and immunoadhesins specifically include all variants disclosed in U.S. Application Publication No. 20050070689. The entire content of U.S. Application Publication No. 20050070689 is hereby expressly incorporated by reference.

3. General Methods for the Recombinant Production of Antibodies

The antibodies and other recombinant proteins herein can be produced by well known techniques of recombinant DNA technology. Thus, aside from the antibodies specifically iden-

tified above, the skilled practitioner could generate antibodies directed against an antigen of interest, e.g., using the techniques described below.

Antigen Selection and Preparation

The antibody herein is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those proteins described in section (3) below. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20, CD22, CD34, CD40; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and $\alpha v/\beta 3$ integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C, or any of the other antigens mentioned herein. Antigens to which the antibodies listed above bind are specifically included within the scope herein.

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with $\frac{1}{5}$ to $\frac{1}{10}$ the original amount of antigen or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal Antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, Protein A-Sepharose, hydroxyapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. Preferably the Protein A chromatography procedure described herein is used.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by

using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

In a further embodiment, monoclonal antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional hybridoma techniques for isolation of monoclonal antibodies.

Humanized and Human Antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human FR for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immnol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and Duchosal et al., *Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J Mol. Biol.,* 222:581-597 (1991); Vaughan et al., *Nature Biotech* 14:309 (1996)).

Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv) (see WO 93/16185).

Multispecific Antibodies

Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies,

BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994). Alternatively, the antibodies can be "linear antibodies" as described in Zapata et al., *Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

Immunoadhesins

The simplest and most straightforward immunoadhesin design combines the binding domain(s) of the adhesin (e.g. the extracellular domain (ECD) of a receptor) with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the immunoadhesins of the present invention, nucleic acid encoding the binding domain of the adhesin will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, C_H2 and C_H3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the C_H1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics of the immunoadhesin.

In a preferred embodiment, the adhesin sequence is fused to the N-terminus of the Fc domain of immunoglobulin G_1 (IgG_1). It is possible to fuse the entire heavy chain constant region to the adhesin sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (i.e. residue 216, taking the first residue of heavy chain constant region to be 114), or analogous sites of other immunoglobulins is used in the fusion. In a particularly preferred embodiment, the adhesin amino acid sequence is fused to (a) the hinge region and C_H2 and C_H3 or (b) the C_H1, hinge, C_H2 and C_H3 domains, of an IgG heavy chain.

For bispecific immunoadhesins, the immunoadhesins are assembled as multimers, and particularly as heterodimers or heterotetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of four basic units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each of the four units may be the same or different.

Various exemplary assembled immunoadhesins within the scope herein are schematically diagrammed below:

AC_L-AC_L;

AC_H-(AC_H, AC_L-AC_H, AC_L-V_HC_H, or V_LC_L-AC_H);

AC_L-AC_H-(AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H)

AC_L-V_HC_H-(AC_H, or AC_L-V_HC_H, or V_LC_L-AC_H);

V_LC_L-AC_H-(AC_L-V_HC_H, or V_LC_L-AC_H); and

$(A\text{-}Y)_n$-$(V_LC_L\text{-}V_HC_H)_2$;

wherein each A represents identical or different adhesin amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_H is an immunoglobulin heavy chain constant domain;

n is an integer greater than 1;

Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown.

However, where such domains are required for binding activity, they shall be constructed to be present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the adhesin sequences can be inserted between immunoglobulin heavy chain and light chain sequences, such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the adhesin sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the C_H2 domain, or between the C_H2 and C_H3 domains. Similar constructs have been reported by Hoogenboom, et al., *Mol. Immunol.* 28:1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an adhesin-immunoglobulin heavy chain fusion polypeptide, or directly fused to the adhesin. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the adhesin-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Pat. No. 4,816,567, issued 28 Mar. 1989.

Immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the adhesin portion in-frame to an immunoglobulin cDNA sequence. However, fusion to genomic immunoglobulin fragments can also be used (see, e.g. Aruffo et al., *Cell* 61:1303-1313 (1990); and Stamenkovic et al., *Cell* 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain

constant regions can be isolated based on published sequences from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the "adhesin" and the immunoglobulin parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells.

Further details of the invention are provided in the following non-limiting Examples.

All patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties.

EXAMPLES

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC® accession numbers is the American Type Culture Collection, Manassas, Va.

Example 1

Production of Polypeptides in Glutamine-Free Production Medium

Materials and Methods:

Cell Lines.

In these studies, CHO host cells expressing an Apomab antibody, anti-VEGF antibody, and the fusion protein BR3-Fc, respectively were used. The host cells were adapted in suspension and serum free cultures. Frozen stocks were prepared as master or working cell banks in the media described below.

Cell line maintenance was carried out using a 250-mL or 1-Liter Corning® vented shake flasks maintained in a Thermo Scientific Forma® reach-in a CO_2 humidified incubator maintained at 37° C. and 5% CO_2. Flasks were agitated at rate of 150 rpm on a New Brunswick Scientific Innova®-2100 platform shaker with a custom aluminum-substrate platform. Cell cultures were passed every 3 or 4 days with fresh media and seeded at 0.11% or 0.20% Packed Cell Volume (PCV). PCV was obtained using a glass 10-mL KIMAX® USA PCV tube.

Culture Media and Conditions.

Media studies were initiated using 250-mL Corning vented shake flask inoculated in singlet, duplicate, or triplicate at 100 mL working volume at 0.20% PCV for all cases using cell culture from a source1-Liter Corning® vented shake flask with a 500-mL working volume. PCV was obtained using a glass 10-mL KIMAX® USA PCV tube.

Prior to initiation of the study cell culture was centrifuged at 1000 rpm for 5-minutes in a Sorvall® RT 6000B centrifuge to complete a 100% media exchange of inoculum media containing glutamine with the respective test media. Different concentrations of Glutamine, Glutamate, Asparagine and Aspartate were evaluated in the different test media. The following concentrations were tested: Glutamine 0-10 mM, Glutamate 1-10 mM, Asparagine 0-15 mM, Aspartate 1-10 mM. Media conditions were evaluated in full factorial DOE studies.

The effect of Glutamine-free medium on was also tested in commercially available DMEM/F12 medium. The medium

was used at 5× concentration (7.05 g/L) with extra Asparagine (10 mM total), Aspartate (10 mM total), Glutamine (10 mM total for the Glutamine-containing medium), Glutamate (1 mM total), and glucose (8 g/L total). Glutamine-free and Glutamine-containing medium were compared using Apomab and anti-VEGF antibody expressing cells.

Shake flasks were maintained in a Thermo Scientific Forma® reach-in a CO_2 humidified incubator maintained at 37° C. and 5% CO_2. Flasks were agitated at rate of 150 rpm on a New Brunswick Scientific Innova®-2100 platform shaker with a custom aluminum-substrate platform.

The medium used contained the following components:

Organic Salts and Trace Elements
Ammonium Paramolybdate, Tetrahydrate
Ammonium Vanadium Oxide
Calcium Chloride, Anhydrous
Cupric Sulfate, Pentahydrate
Ferrous Sulfate, Heptahydrate
Potassium Chloride
Magnesium Chloride, Anhydrous
Manganese Sulfate, Monohydrate
Nickel Chloride, Hexahydrate
Selenious Acid
Sodium Metasilicate, Nonahydrate
Sodium Phosphate, Monobasic, Monohydrate
Stannous Chloride, Dihydrate
Zinc Sulfate, Heptahydrate
Lipids
Linoleic Acid
Lipoic Acid (aka Thioctic Acid)
Putrescine, Dihydrochloride
Amino Acids
L-Alanine
L-Arginine, Monohydrochloride
L-Asparagine
L-Aspartic Acid
L-Cysteine, Monohydrochloride, Monohydrate
L-Glutamic Acid
L-Glutamine
L-Histidine, Monohydrochloride, Monohydrate
L-Isoleucine
L-Leucine
L-Lysine, Monohydrochloride
L-Methionine
L-Phenylalanine
L-Proline
L-Serine
L-Threonine
L-Tryptophan
L-Tyrosine, Disodium Salt, Dihydrate
L-Valine
Vitamins
Biotin
D-Calcium Pantothenate
Choline Chloride
Folic Acid
I-Inositol
Niacinamide
Pyridoxine, Monohydrochloride
Riboflavin
Thiamine, Monohydrochloride
Vitamin B-12
Carbon Source, Growth Factors, and Miscellaneous
Fluronic F-68
D-Glucose
Sodium Bicarbonate
Sodium Pyruvate
Sodium Chloride
Sodium Hydroxide
Insulin
Galactose

The commercially-available DMEM/F-12 culture medium was also tested, having the following components;

	(mg/L)
VITAMINS	
Biotin	0.00365
D-calcium pantothenate	2.24
Choline chloride	8.98
Cyanocobalamin	0.68
Folic acid	2.65
i-inositol	12.6
Niacinamide	2.0185
Pyridoxal HCl	2
Pyridoxine HCI	0.031
Riboflavin	0.219
Thiamine HCl	2.17
AMINO ACIDS	
L-alanine	4.455
L-arginine HCl	147.5
L-asparagine monohydrate	7.5
L-aspartic acid	6.65
L-cysteine HCI monohydrate	17.56
L-cystine 2HCl	31.29
L-glutamic acid	7.35
L-glutamine	365
Glycine	18.75
L-histidine HCl monohydrate	31.48
L-isoleucine	54.47
L-leucine	59.05
L-lysine HCl	91.25
L-methionine	17.24
L-phenylalanine	35.48
L-proline	17.25
L-serine	26.25
L-threonine	53.45
L-tryptophan	9.02
L-tyrosine 2Na dihydrate	55.79
L-valine	52.85
OTHER	
Dextrose anhydrous	3151
HEPES	3575
Hypoxanthine sodium salt	2.39
Linoleic acid	0.042
DL-α-Lipoic acid	0.105
Phenol red sodium salt	8.602
Putrescine 2HCI	0.081
Sodium pyruvate	55
Thymidine	0.365
ADD: Sodium bicarbonate	1200
INORGANIC SALTS	
Calcium chloride anhydrous	116.61
Cupric sulfate pentahydrate	0.00125
Ferric nitrate nonahydrate	0.05
Ferrous sulfate heptahydrate	0.417
Magnesium chloride anhydrous	28.61
Magnesium sulfate anhydrous	48.84
Potassium chloride	311.8
Sodium chloride	6999.5
Sodium phosphate dibasic anhydrous	71.02
Sodium phosphate monobasic monohydrate	62.5
Zinc sulfate heptahydrate	0.4315

The medium for inoculum culture (as opposed for the production phase) was usually supplemented with 5 mM glutamine, 8 g/L glucose, and 75-2000 nM Methotroxate.

For studies pH adjustment was performed as needed to maintain pH value at 7.00±0.10 using 1M Sodium Carbonate.

Adjustment in pH value was made in by adding 1 mL/L of 1M Sodium Carbonate to raise pH units up 0.10.

Cell culture was analyzed up to 14-days by taking a 3.5-mL sample and analyzed for viable cell count, viability, and cell size using a Beckman Coulter ViCell™-1.0 cell counter. Nutrient analysis was performed using the Nova 400 Biomedical Bioprofile®. Osmolality was measured using an Advanced® Instrument multi-sample Osmometer (Model 3900). Recombinant product titer concentration was obtained using the Agilent 1100 Series HPLC.

Recombinant Proteins.

The recombinant proteins produced were Apomab (TRAIL), anti-VEGF, and the immunoadhesin BR3-Fc.

Data Analysis.

Statistical analyses of the data were carried out using a full factorial design of experiment, which is an experiment whose design consists of two or more factors, each with discrete possible values or "levels", and whose experimental units take on all possible combinations of these levels across all such factors. A full factorial design may also be called a fully-crossed design. Such an experiment allows studying the effect of each factor on the response variable, as well as the effects of interactions between factors on the response variable.

Results

As shown in FIGS. **1-5**, use of a glutamine-free production medium increased the final recombinant protein titer of Apomab antibody, BR3-Fc immunoadhesin and anti-VEGF antibody. In each case, cube plot analysis of titer results using Full Factorial DOE evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate predict that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid. (FIGS. **1-3**)

The effect of Asparagine under Glutamine-free, low Glutamate and high Aspartate conditions on Apomab antibody titer is shown in FIG. **4**. In Glutamine-free medium, Apomab antibody titer was significantly increased in the presence of 2.5-15 mM Asparagine compared to Glutamine-free cultures without Asparagine. Under these conditions, the presence or absence of Glutamate had no effect on titer.

Apomab antibody titer production across various Asparagine and Aspartate concentrations in Glutamine-free and low Glutamate conditions is illustrated in FIG. **5**. A positive titration effect was observed when increasing Aspartate from 0 to 10 mM under these conditions.

The effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on titer is demonstrated in FIGS. **6**A-C, wherein the final titer for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin (A-C, respectively) was significantly higher in Glutamine-free medium compared to Glutamine-containing medium.

Similar results were obtained using the commercial DMEM/F-12 culture medium. As shown in FIGS. **7**A and B, the final titer for Apomab antibody and anti-VEGF antibody (A and B, respectively) was significantly higher in Glutamine-free DMEM/F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing DMEM F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

As shown in FIGS. **8** and **9**, use of a glutamine-free production medium also increased specific production measured as Qp (mg/mL-cell/day). FIGS. **8** A-C illustrate that cell specific productivity (Qp) for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin (A-C, respectively)

was significantly higher in Glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing medium. FIGS. **9**A and B illustrate that cell specific productivity for Apomab antibody and anti-VEGF antibody (A and B, respectively) was significantly higher in Glutamine-free DMEM/F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing DMEM/F12 medium.

As shown in FIGS. **10** and **11**, use of a glutamine-free production medium was shown to improve cell viability and extend culture longevity significantly. FIGS. **10**A-C. illustrate that cell viability for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin (A-C, respectively) was higher in Glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing medium. FIGS. **11**A and B indicate that, in DMEM/F12 medium, cell viability was not consistently improved in Glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid. Of note, viability was higher for Apomab antibody (FIG. **11**A), but lower for anti-VEGF antibody (FIG. **11**B) compared to Glutamine containing medium.

As shown in FIGS. **12** and **13**, use of a glutamine-free production medium reduced NH_4^+ accumulation significantly compared to glutamine-containing medium. FIGS. **12** A-C illustrate that ammonia levels were usually lower in Glutamine-free cultures supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing cultures. FIGS. **13** A and B illustrate that ammonia levels were significantly reduced in Glutamine-free DMEM/F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing DMEM/F12 medium.

The invention illustratively described herein can suitably be practiced in the absence of any element or elements, limitation or limitations that is not specifically disclosed herein. Thus, for example, the terms "comprising," "including," "containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalent of the invention shown or portion thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modifications and variations of the inventions embodied herein disclosed can be readily made by those skilled in the art, and that such modifications and variations are considered to be within the scope of the inventions disclosed herein.

From the description of the invention herein, it is manifest that various equivalents can be used to implement the concepts of the present invention without departing from its scope. Moreover, while the invention has been described with specific reference to certain embodiments, a person of ordinary skill in the art would recognize that changes can be made in form and detail without departing from the spirit and the scope of the invention. The described embodiments are considered in all respects as illustrative and not restrictive. It should also be understood that the invention is not limited to the particular embodiments described herein, but is capable of many equivalents, rearrangements, modifications, and substitutions without departing from the scope of the invention.

Thus, additional embodiments are within the scope of the invention and within the following claims.

All U.S. patents and applications; foreign patents and applications; scientific articles; books; and publications mentioned herein are hereby incorporated by reference in their entirety as if each individual patent or publication was specifically and individually indicated to be incorporated by reference, including any drawings, figures and tables, as though set forth in full.

What is claimed is:

1. A process for producing a polypeptide in a mammalian host cell expressing said polypeptide, comprising culturing the mammalian host cell in a production phase of the culture in a glutamine-free production culture medium containing asparagine, wherein the asparagine is added at a concentration in the range of 7.5 mM to 15 mM.

2. The process of claim **1** wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

3. The process of claim **1** wherein said recombinant host cell is an eukaryotic host cell.

4. The process of claim **3** wherein said eukaryotic host cell is a Chinese Hamster Ovary (CHO) cell.

5. The process of claim **4** wherein the mammalian host cell is a dhfr$^-$CHO cell.

6. The process of claim **1** wherein the production medium is serum-free.

7. The process of claim **1** wherein the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

8. The process of claim **7** wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

9. The process of claim **1** wherein the production phase is a batch or fed batch culture phase.

10. The process of claim **1** further comprising the step of isolating said polypeptide.

11. The process of claim **10** further comprising determining one or more of cell viability, culture longevity, specific productivity and final recombinant protein titer following isolation.

12. The process of claim **11** wherein at least one of the cell viability, culture longevity, specific productivity and final recombinant protein titer is increased relative to the same polypeptide produced in a glutamine-containing production medium of the same composition.

13. The process of claim **1** wherein the polypeptide is a mammalian glycoprotein.

14. The process of claim **1** wherein the polypeptide is selected from the group consisting of antibodies, antibody fragments, and immunoadhesins.

15. The process of claim **14** wherein said antibody fragment is selected from the group consisting of Fab, Fab', F(ab')2, scFv, (scFv)2, dAb, complementarity determining region (CDR) fragments, linear antibodies, single-chain antibody molecules, minibodies, diabodies, and multispecific antibodies formed from antibody fragments.

16. The process of claim **14** wherein the antibody or antibody fragment is chimeric, humanized or human.

17. The process of claim **14** wherein said antibody or antibody fragment is a therapeutic antibody or a biologically functional fragment thereof.

18. The process of claim **17** wherein said therapeutic antibody is selected from the group consisting of anti-HER2 antibodies anti-CD20 antibodies; anti-IL-8 antibodies; anti-VEGF antibodies; anti-CD40 antibodies, anti-CD11a antibodies; anti-CD18 antibodies; anti-IgE antibodies; anti-Apo-2 receptor antibodies; anti-Tissue Factor (TF) antibodies; anti-human $\alpha 4\beta 7$ integrin antibodies; anti-EGFR antibodies; anti-CD3 antibodies; anti-CD25 antibodies; anti-CD4 antibodies; anti-CD52 antibodies; anti-Fc receptor antibodies; anti-carcinoembryonic antigen (CEA) antibodies; antibodies directed against breast epithelial cells; antibodies that bind to colon carcinoma cells; anti-CD38 antibodies; anti-CD33 antibodies; anti-CD22 antibodies; anti-EpCAM antibodies; anti-GpIIb/IIIa antibodies; anti-RSV antibodies; anti-CMV antibodies; anti-HIV antibodies; anti-hepatitis antibodies; anti-CA 125 antibodies; anti-$\alpha v\beta 3$ antibodies; anti-human renal cell carcinoma antibodies; anti-human 17-1A antibodies; anti-human colorectal tumor antibodies; anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma; and anti-human leukocyte antigen (HLA) antibodies, and anti-HLA DR antibodies.

19. The process of claim **17** wherein said therapeutic antibody is an antibody binding to a HER receptor, VEGF, IgE, CD20, CD11a, CD40, BR3 or DR5.

20. The process of claim **19** wherein said therapeutic antibody binding to DR5 is selected from the group consisting of Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3.

21. The process of claim **19** wherein said therapeutic antibody is an anti-BR3 antibody.

22. The process of claim **14** wherein said immunadhesin is a BR3-Fc immunoadhesin.

23. The process of claim **1** wherein said polypeptide is a therapeutic polypeptide.

24. The process of claim **23** wherein said therapeutic polypeptide is selected from the group consisting of a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal

growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of said polypeptides.

25. A ready-to-use glutamine-free cell culture medium containing asparagine for the production of a polypeptide in a production phase, wherein the asparagine is at a concentration in the range of 7.5 mM to 15 mM.

* * * * *

EXHIBIT CC

US008574869B2

(12) **United States Patent**
Kao et al.

(10) **Patent No.: US 8,574,869 B2**
(45) **Date of Patent: Nov. 5, 2013**

(54) **PREVENTION OF DISULFIDE BOND REDUCTION DURING RECOMBINANT PRODUCTION OF POLYPEPTIDES**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/354,223**

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Related U.S. Application Data

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(60) Provisional application No. 60/948,677, filed on Jul. 9, 2007.

(51) **Int. Cl.**
C12P 1/00 (2006.01)
C12N 5/02 (2006.01)

(52) **U.S. Cl.**
USPC **435/41**; 435/325

(58) **Field of Classification Search**
None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

2004/0029229 A1*	2/2004	Reeves et al.	....	435/69.1
2004/0138424 A1	7/2004	Takeda et al.		
2006/0143549 A1	6/2006	Yasumoto et al.		
2007/0292411 A1	12/2007	Salcedo et al.		
2009/0053786 A1	2/2009	Kao et al.		

FOREIGN PATENT DOCUMENTS

WO	97/26357	7/1997

OTHER PUBLICATIONS

Kock et al. (Biochem Soc Trans. Apr. 2004;32(Pt 2):273-5).*
Christiansen et al. (Biochemistry 1998, 37, 12611-12623).*
Bobovnikova et al., "Characterization of Soluble, Disulfide Bond-Stabilized Prokaryotically Expressed Human Thryotropin Receptor Ectodomain" Endocrinology 138(2):588-593 (1997).
Chaderjian et al., "Effect of Copper Sulfate on Performance of a Serum-Free CHO Cell Culture Process and the Level of Free Thiol in the Recombinant Antibody Expressed" Biotechnology Progress 21(2):550-553 (2005).
Gromer et al., "The Thioredoxin System: From Science to Clinic" Medicubak Research Reviews 24(1):40-89 (Jan. 2004).
Kao et al., "Mechanism of Antibody Reduction in Cell Culture Production Processes" Biotechnology and Bioengineering 107(4):622-632 (Nov. 2010).
Kerblat et al., "Importance of Thioredoxin in the Proteolysis of an Immunoglobulin G as Antigen by Lysosomal Cys-Proteases" Immunology 97(1):62-68 (1999).
Li et al., "Low Level Formation of Potent Catalytic IgG Fragments Mediated by Disulfide Bond Instability" Molecular Immunology 33(7-8):593-600 (1996).
Liu et al., "Study of Tioredoxin" Journal of Northeast Agricultural University 34(23):219-225 (Jun. 30, 2003).
Mun et al., "BIOT 245-Air Sparging of Harvested Cell Culture Fluid (HCCF) to Prevent Antibody Disulfide Bond Reduction" Abstracts of Papers American Chemical Society 238:245 (Aug. 2009).
Nordberg et al., "Reactive Oxygen Species, Antioxidants, and the Mammalian Thioredoxin System" Free Radical Biology & Medicine 31(11):1287-1312 (2001).
Powis et al., "Properties and Biological Activities of Thioredoxins" Annual Review of Pharmacology and Toxicology 41:261-295 (2001).
Powis et al., "Thioredoxin Redox Control of Cell Growth and Death and the Effects of Inhibitors" Chemico-Biologica llnteractions 111-112:23-34 (1998).
Salas-Solano et al., "Optimization and Validation of a Quantitative Capillary Electrophoresis Sodium Dodecyl Sulfate Method for Quality Control and Stability Monitoring of Monoclonal Antibodies" Analytical Chemistry 78(18):6583-6594 (2006).
Smith et al., "Specific Cleavage of Immunoglobulin G by Copper Ions" International Journal of Peptide and Protein Research 48(1):49-55 (1996).
Starks et al., "Atomic-Resolution Crystal Structure of Thioredoxin From the Acidophilic Bacterium Acetobacter Aceti" Protein Science 16(1):92-98 (Jan. 2007).
Teilum et al., "Disulfide Bond Formation and Folding of Plant Peroxidases Expressed as Inclusion Body Protein in *Escherichia coli* Thioredoxin Reductase Negative Strains" Protein Expression and Purification Academic Press 15(1):77-82 (1999).
Trexler-Schmidt et al., "Identification and Prevention of Antibody Disulfide Bond Reduction During Cell Culture Manufacturing" Biotechnology and Bioengineering 160(3):452-461 (2006).
Urig et al., "On the Potential of Thioredoxin Reductase Inhibitors for Cancer Therapy" Seminars in Cancer Biology 16(6):452-465 (2006).
Wipf et al., "New Inhibitors of the Thioredoxin-Thioredoxin Reducatase System Based on a Naphthoquinone Spiroketal Natural Product Lead" Bioorganic & Medicinal Chemistry Letters(11):2637-2641 (2001).
Zhang et al., "Free sulfhydryl in recombinant monoclonal antibodies" Biotechnol. Prog. 18:509-513 (2002).
Lillig, C.H. et al. (Jan. 2007). "Thioredoxin and Related Molecules—From Biology to Health and Disease," *Antioxidants & Redox Signaling* 9(1):25-47.
Lydersen, B.K. et al. (Nov. 1994). "Acid Precipitation of Mammalian Cell Fermentation Broth," *Ann. N.Y. Acad. Sci.* 745:222-231.
Roman, B. et al. (Sep. 2005). "Development of a Robust Clarification Process for MAb Purification," *Case Study presented at the BioProcess International 2005 Conference & Exhibition*, Sep. 19-22, 2005, Boston, MA, four pages particularly p. 4.
Roush, D.J. et al. (2008). "Advances in Primary Recovery: Centrifugation and Membrane Technology," *Biotechnol. Prog.* 24(3):488-495.

* cited by examiner

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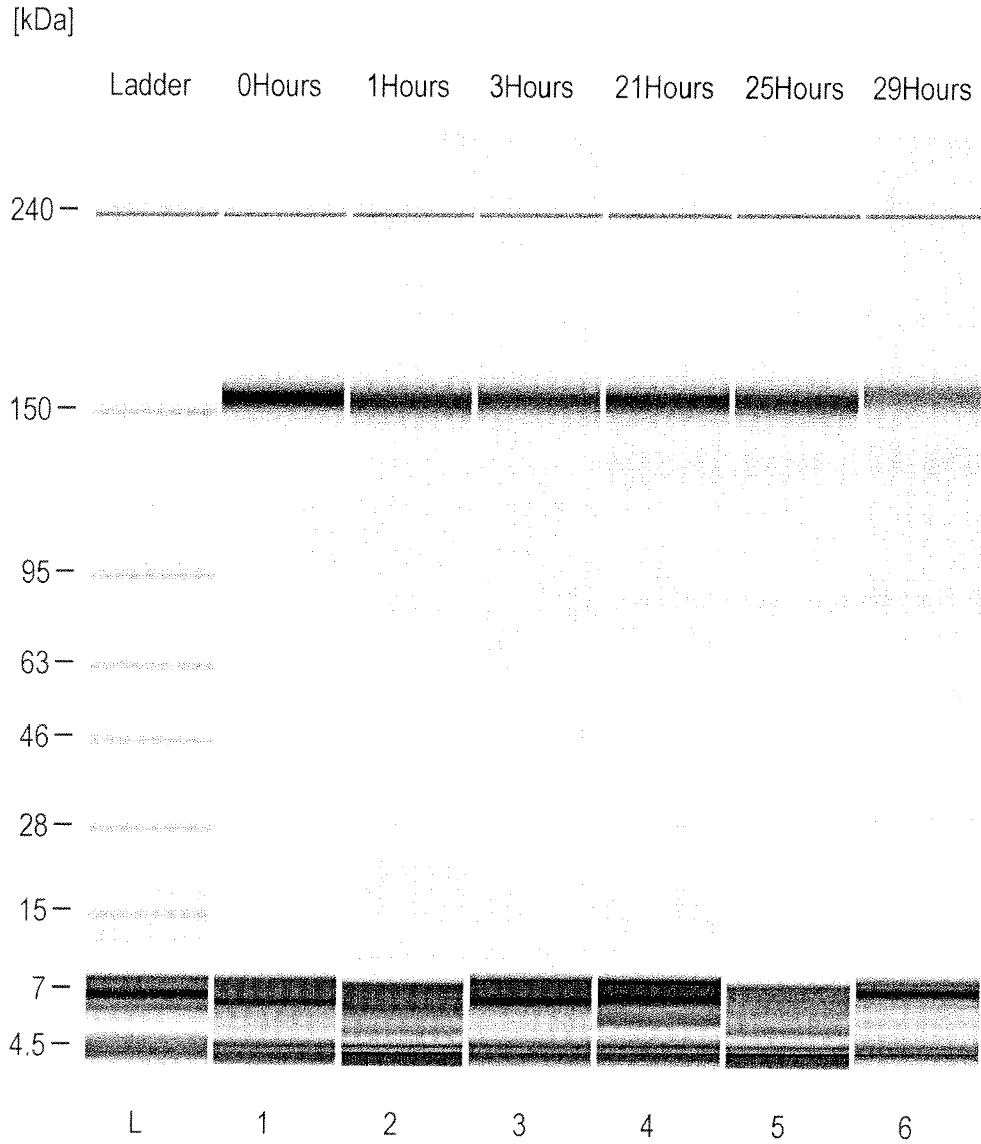
(74) *Attorney, Agent, or Firm* — Morrison & Foerster LLP

(57) **ABSTRACT**

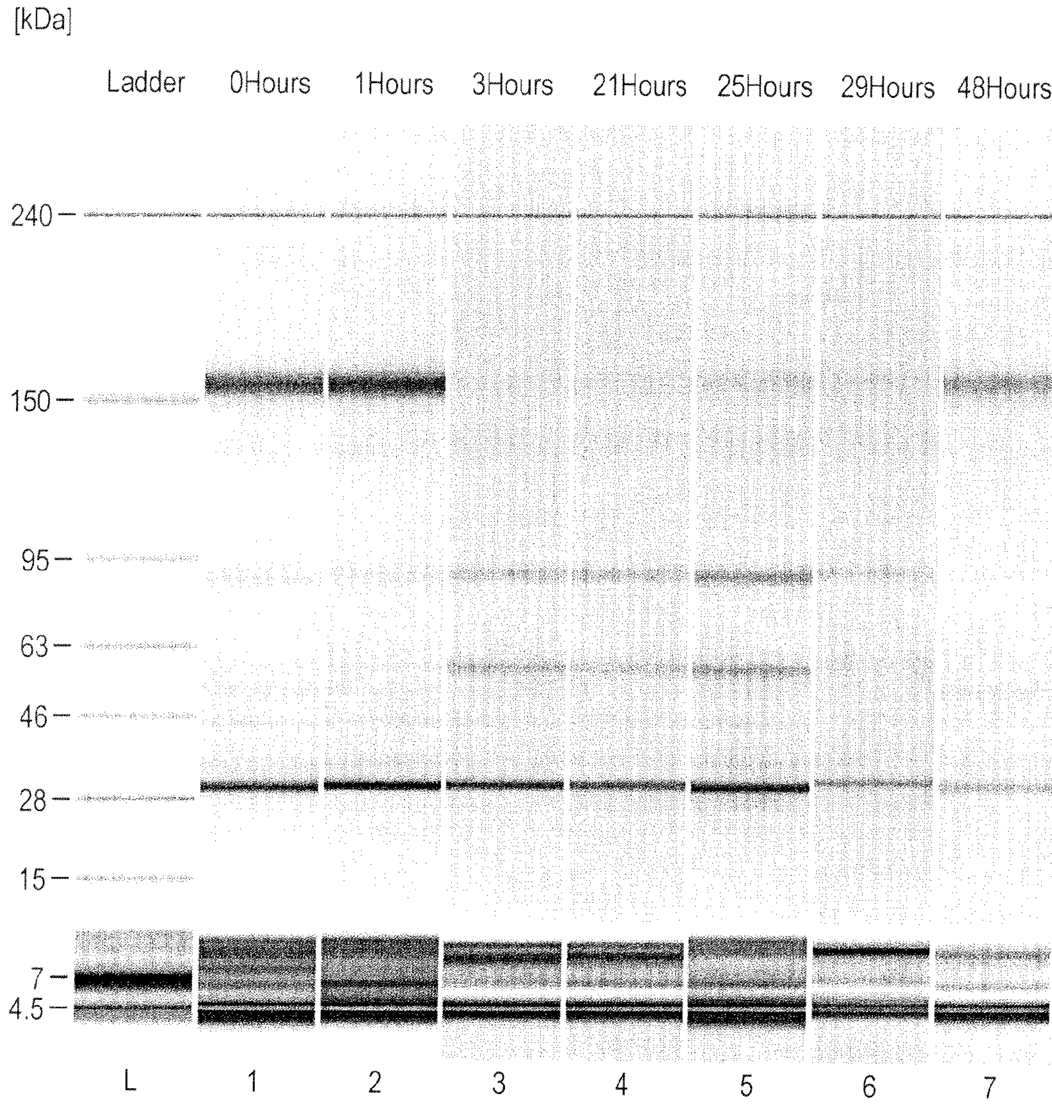
Provided herein are methods for preventing the reduction of disulfide bonds during the recombinant production of disulfide-containing polypeptides. In particular, the invention concerns the prevention of disulfide bond reduction during harvesting of disulfide-containing polypeptides, including antibodies, from recombinant host cell cultures.

10 Claims, 40 Drawing Sheets



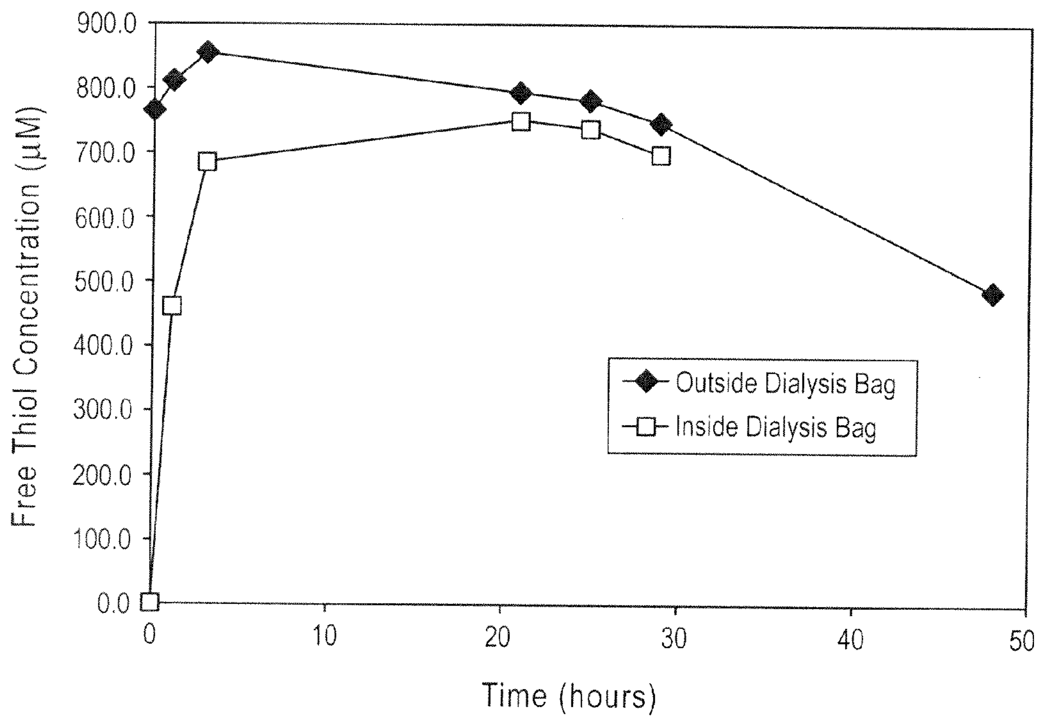
Dialysis Experiment

FIG. 1



Dialysis Experiment

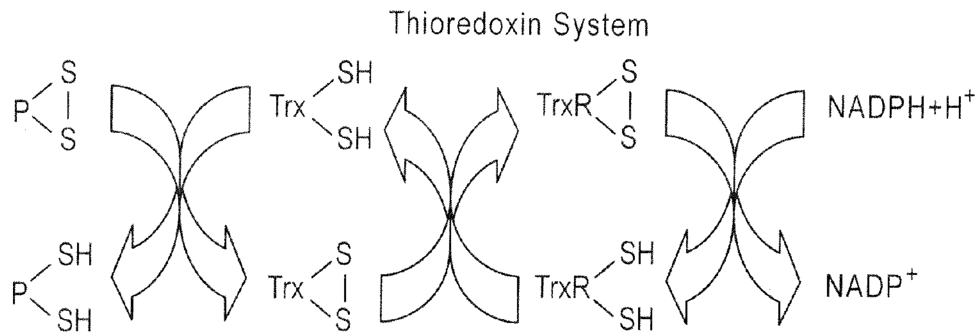
FIG. 2



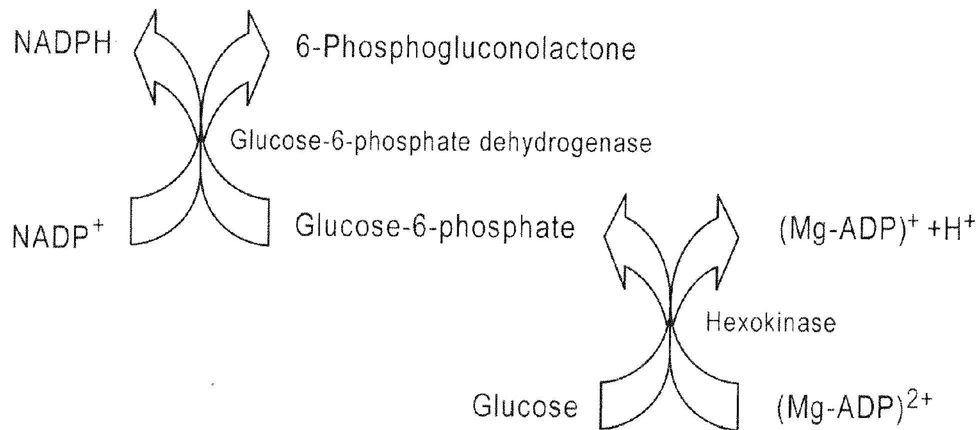
Free Thiol Levels from Dialysis Experiment

FIG. 3

Thioredoxin System



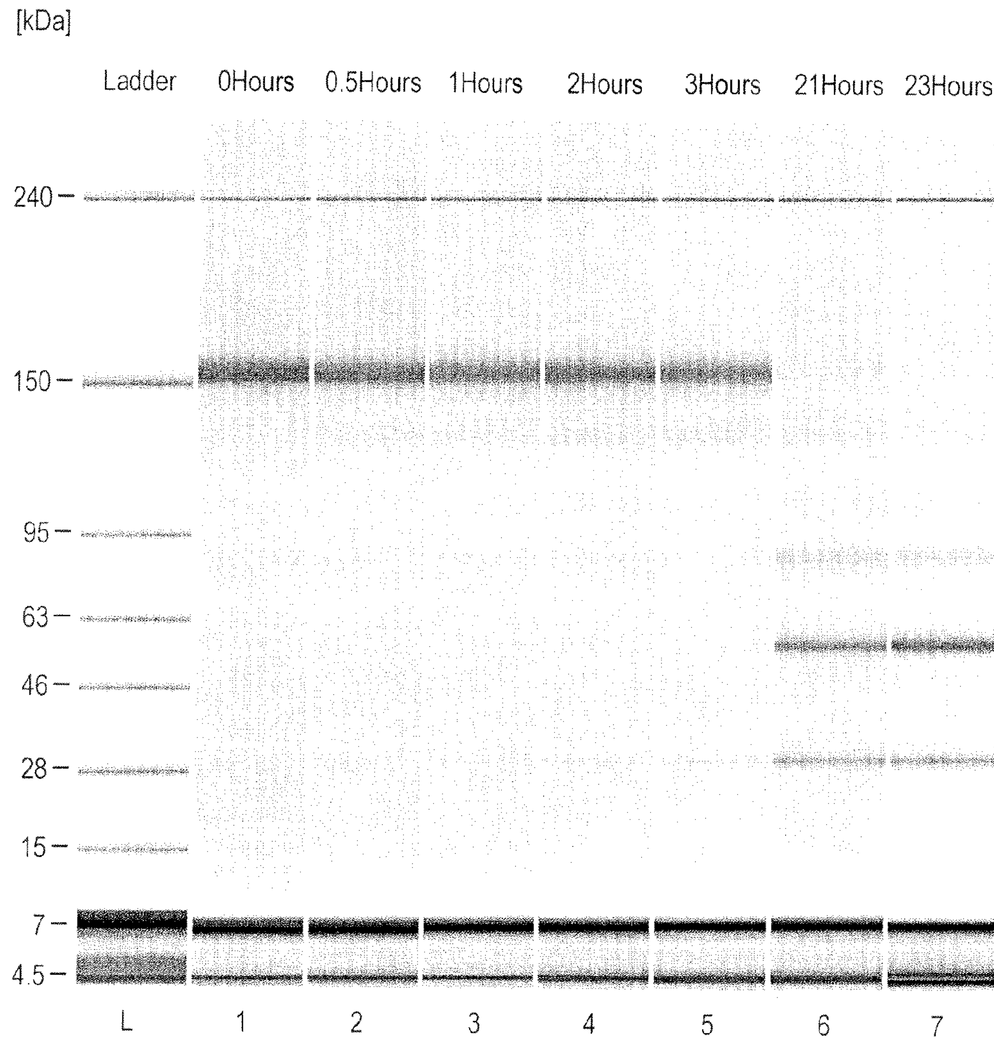
First Reaction in Pentose Phosphate Pathway



First Reaction in Glycolysis

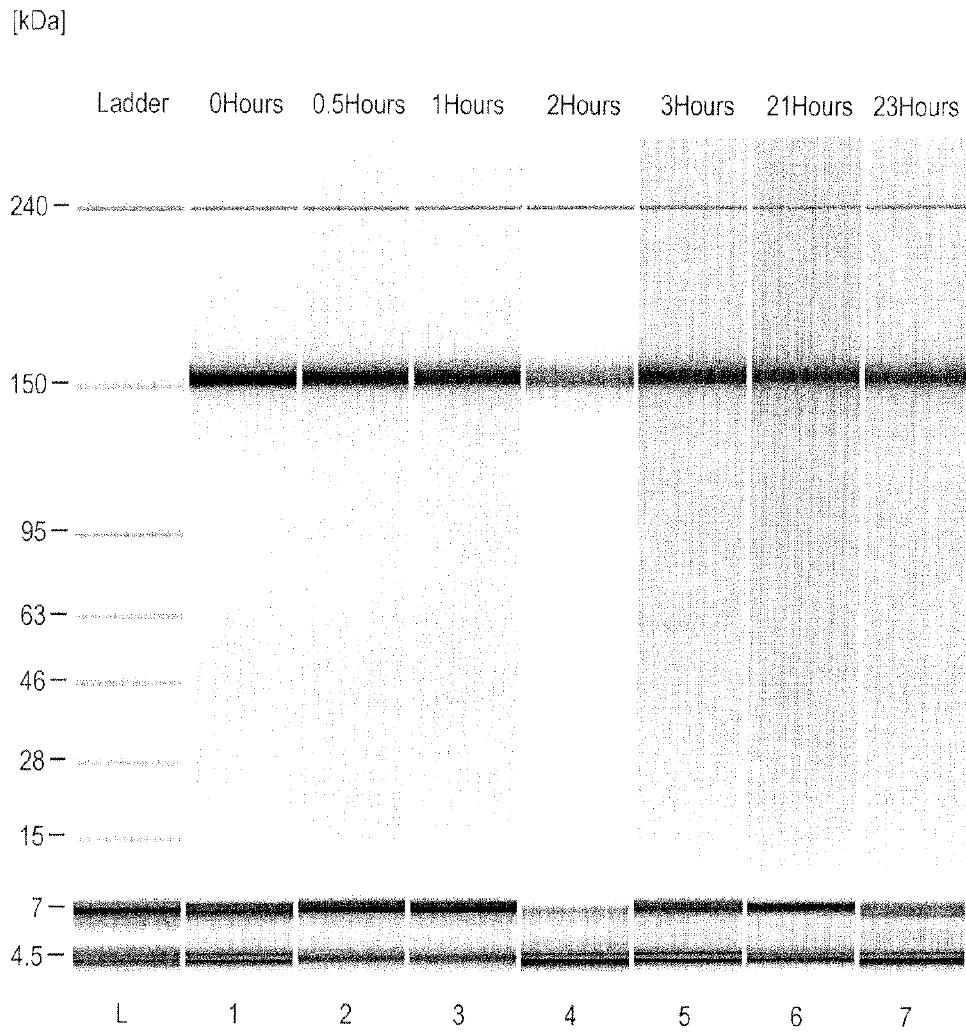
Thioredoxin System and Other Reactions Involved in Antibody Reduction

FIG. 4



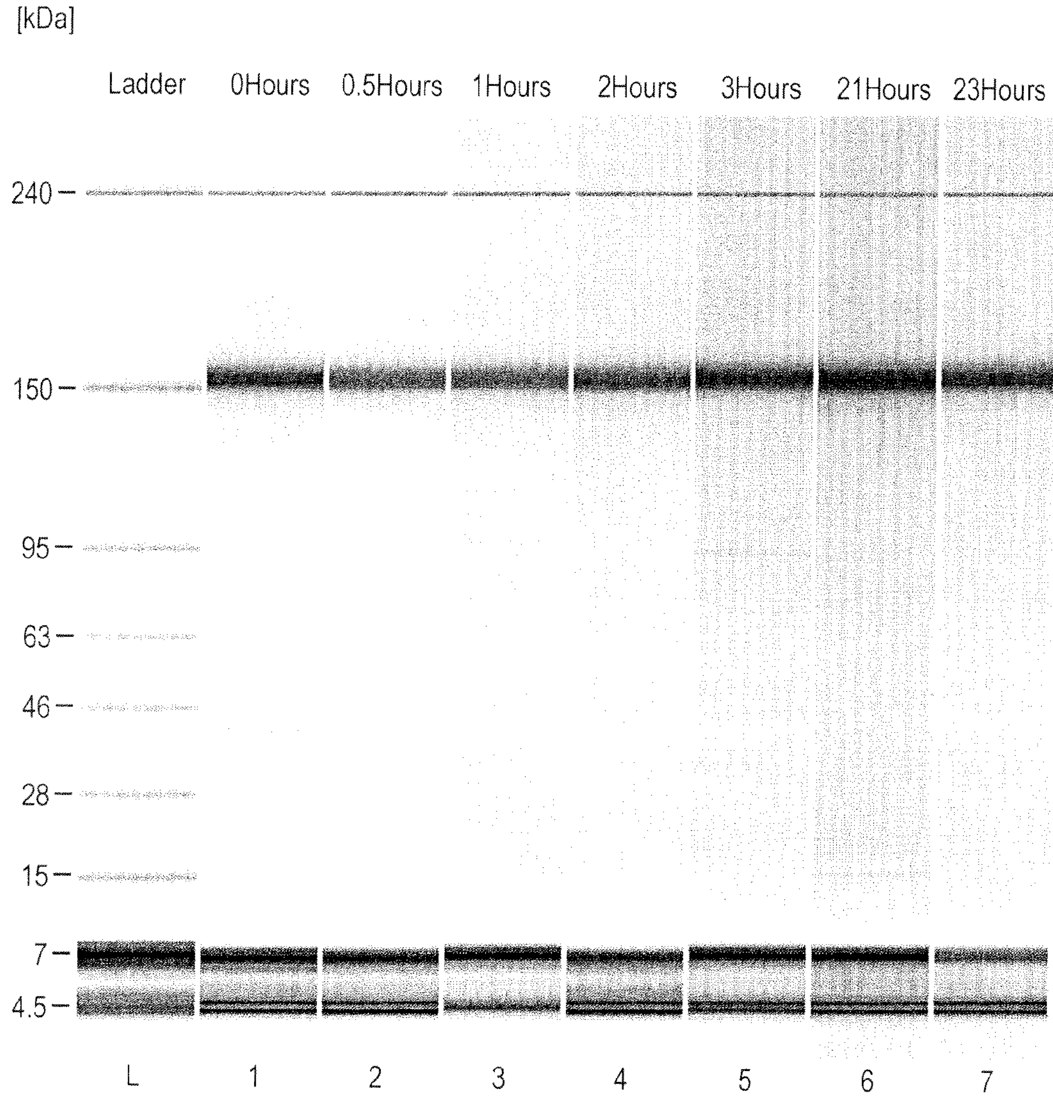
In *Vitro* Activity of Thioredoxin System

FIG. 5



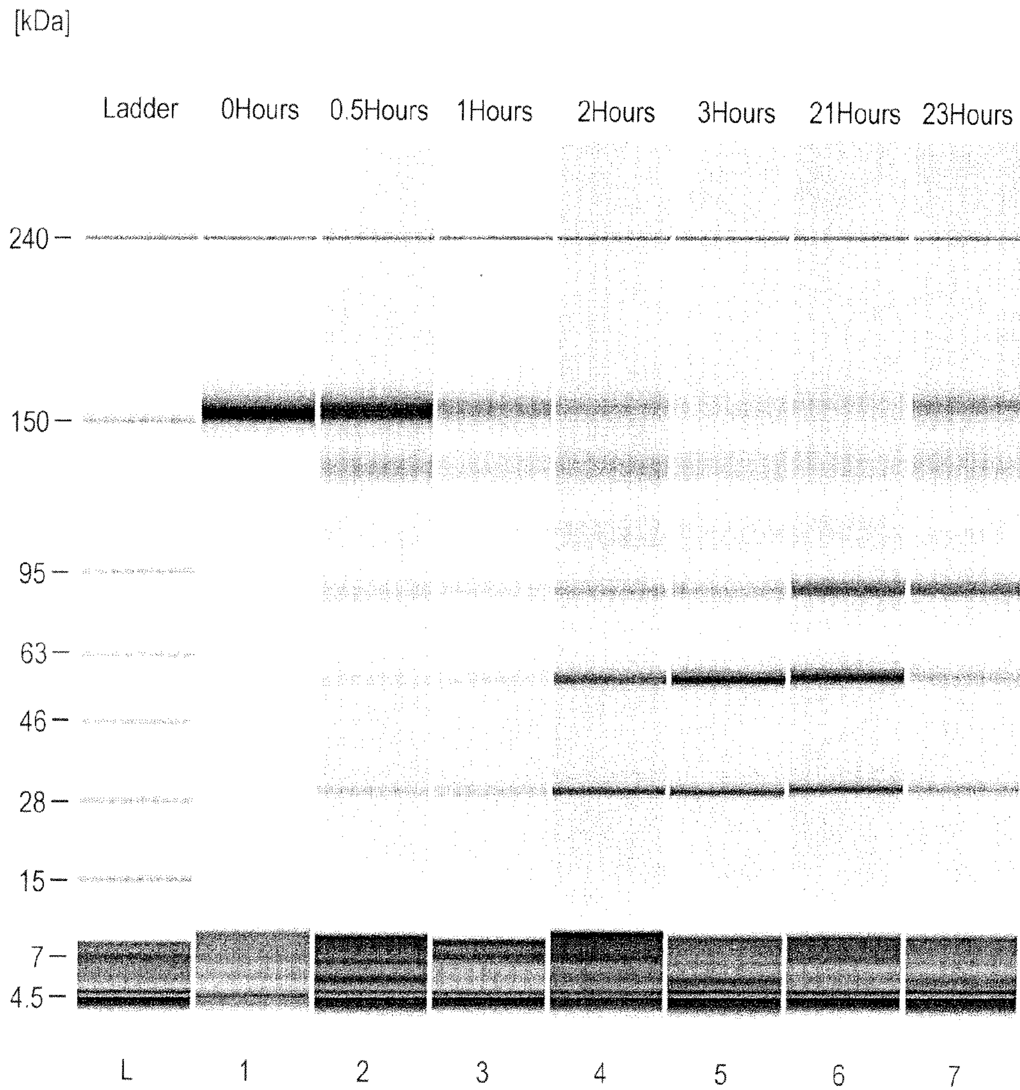
In *vitro* Activity of Thioredoxin System Inhibited by Aurothioglucose

FIG. 6



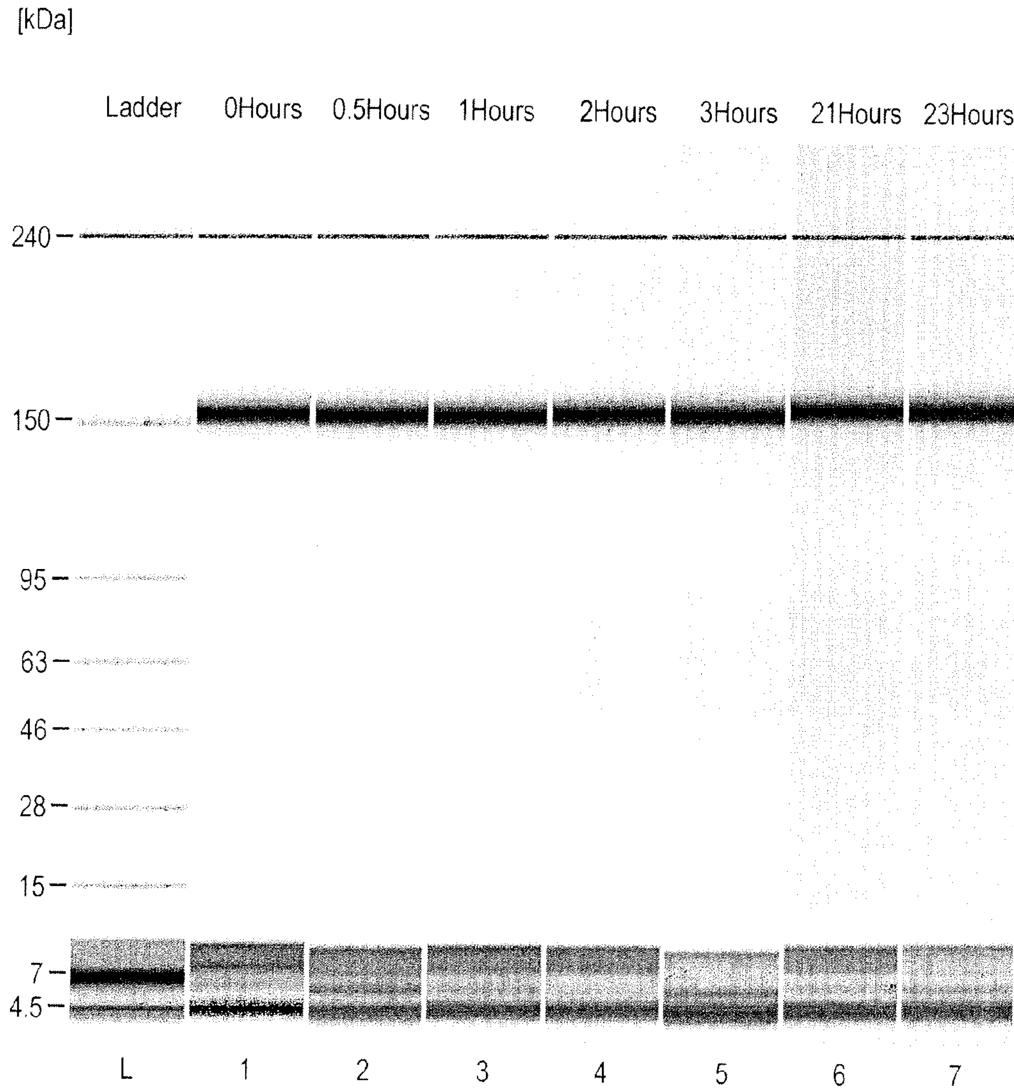
In *vitro* Activity of Thioredoxin System Inhibited by Aurothiomalate

FIG. 7



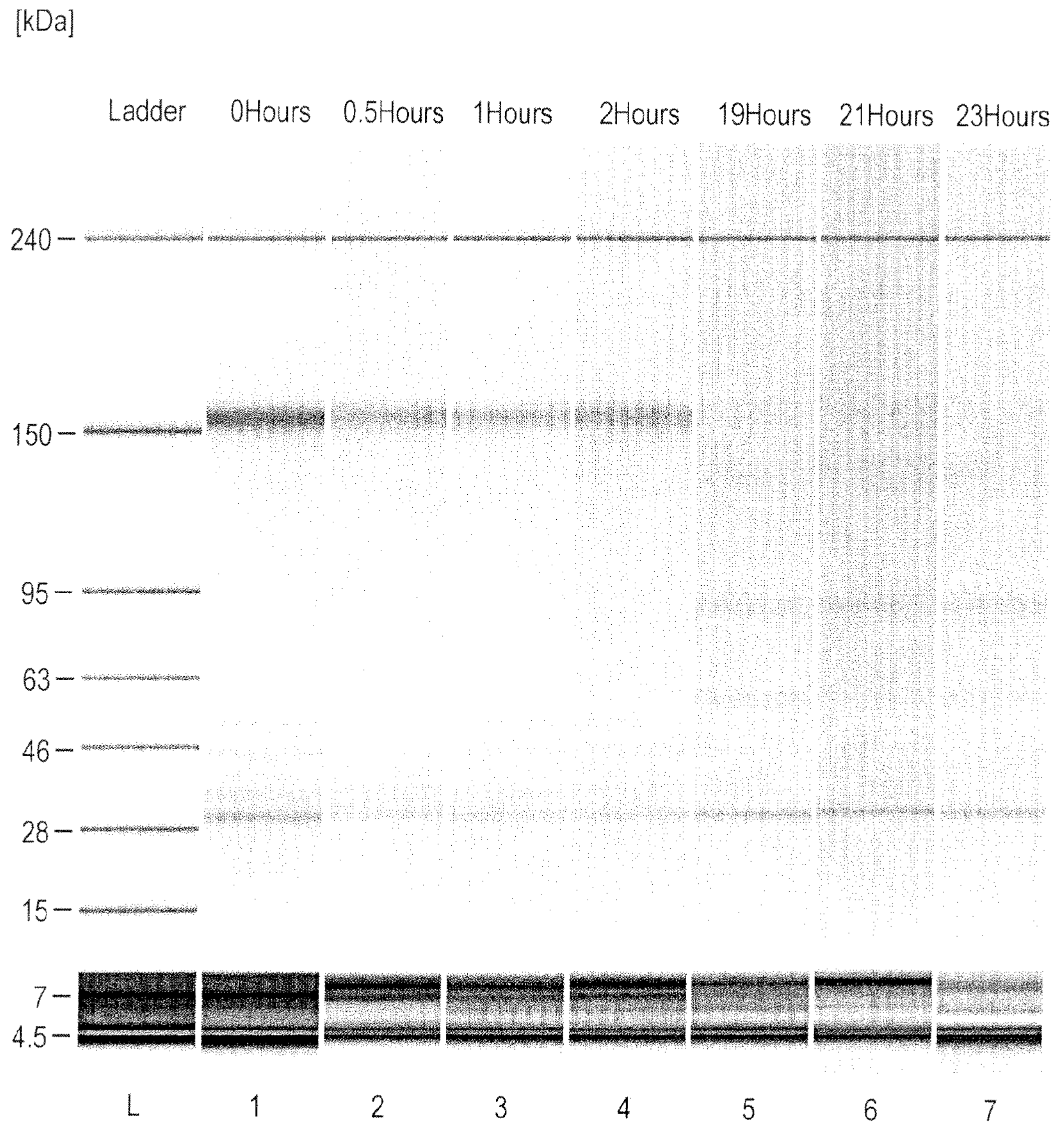
In *vitro* Activity of Thioredoxin System

FIG. 8



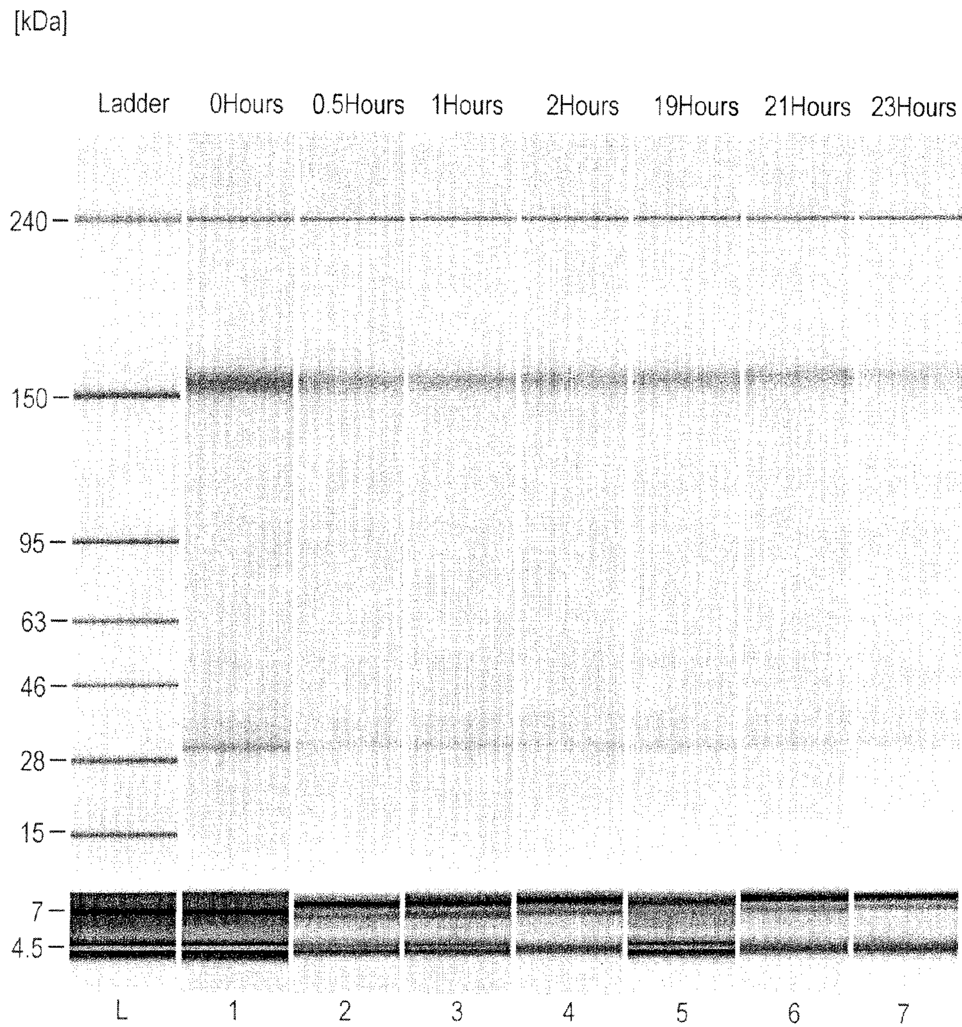
In *vitro* Activity of Thioredoxin System Inhibited by $CuSO_4$

FIG. 9



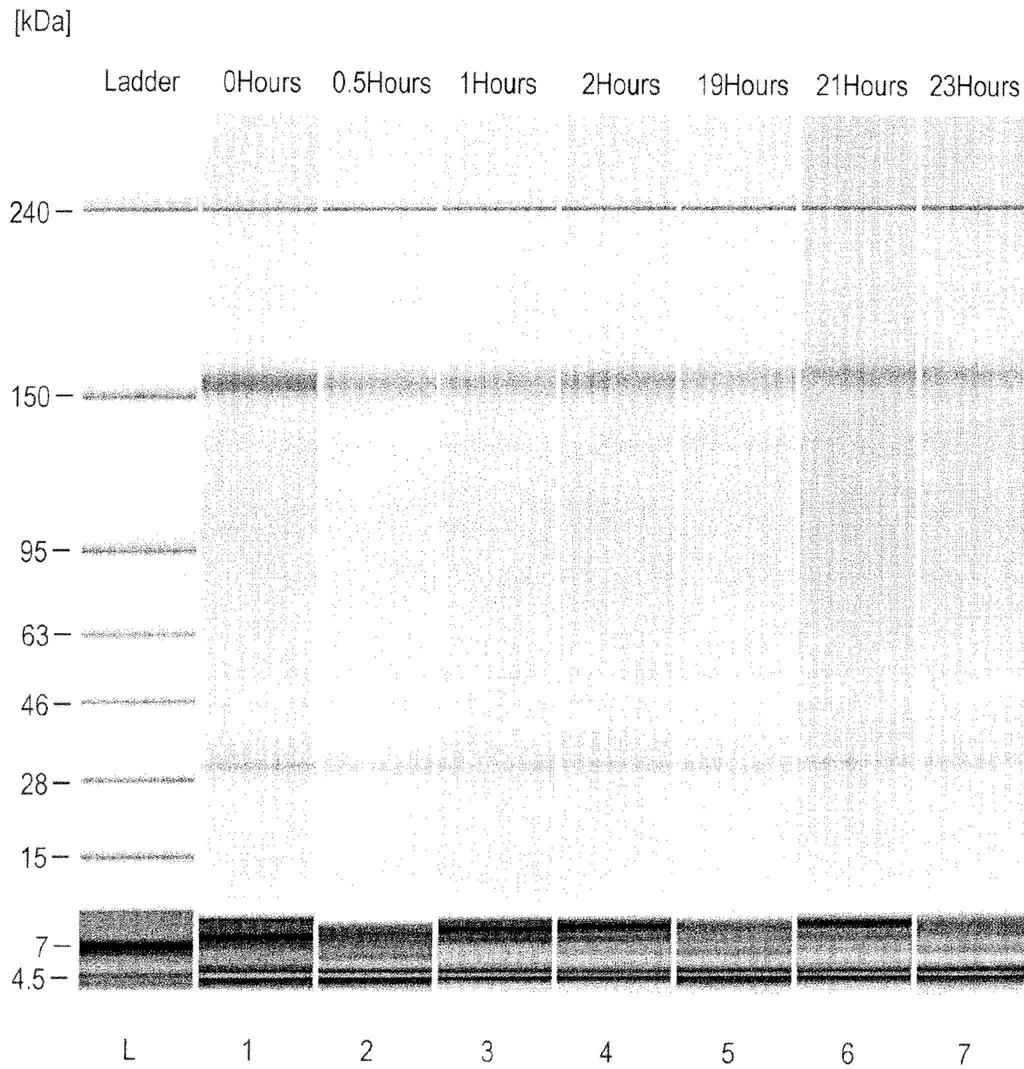
Ocrelizumab Reduction

FIG. 10



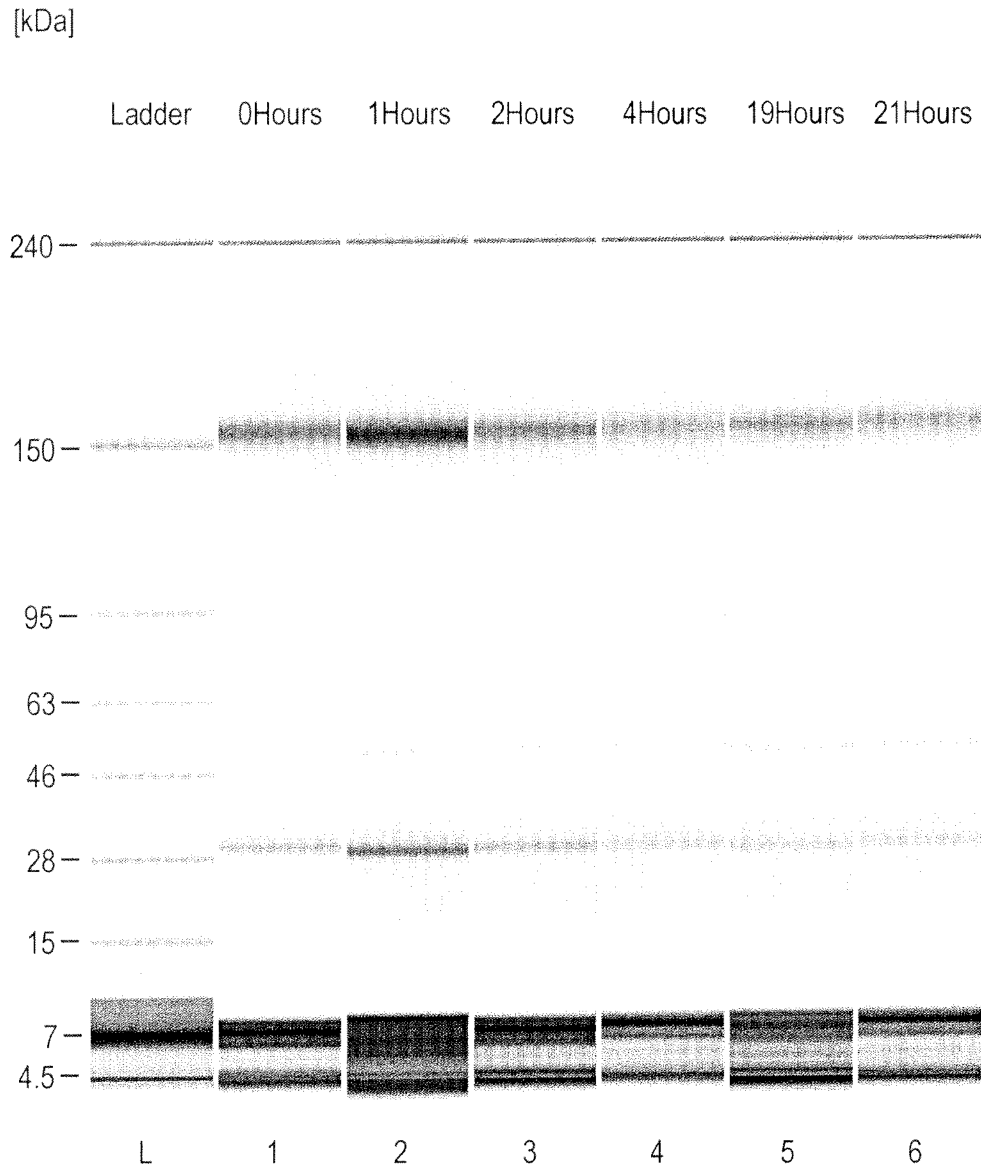
Inhibition of Ocrelizumab Reduction In HCCF by Aurothioglucose

FIG. 11



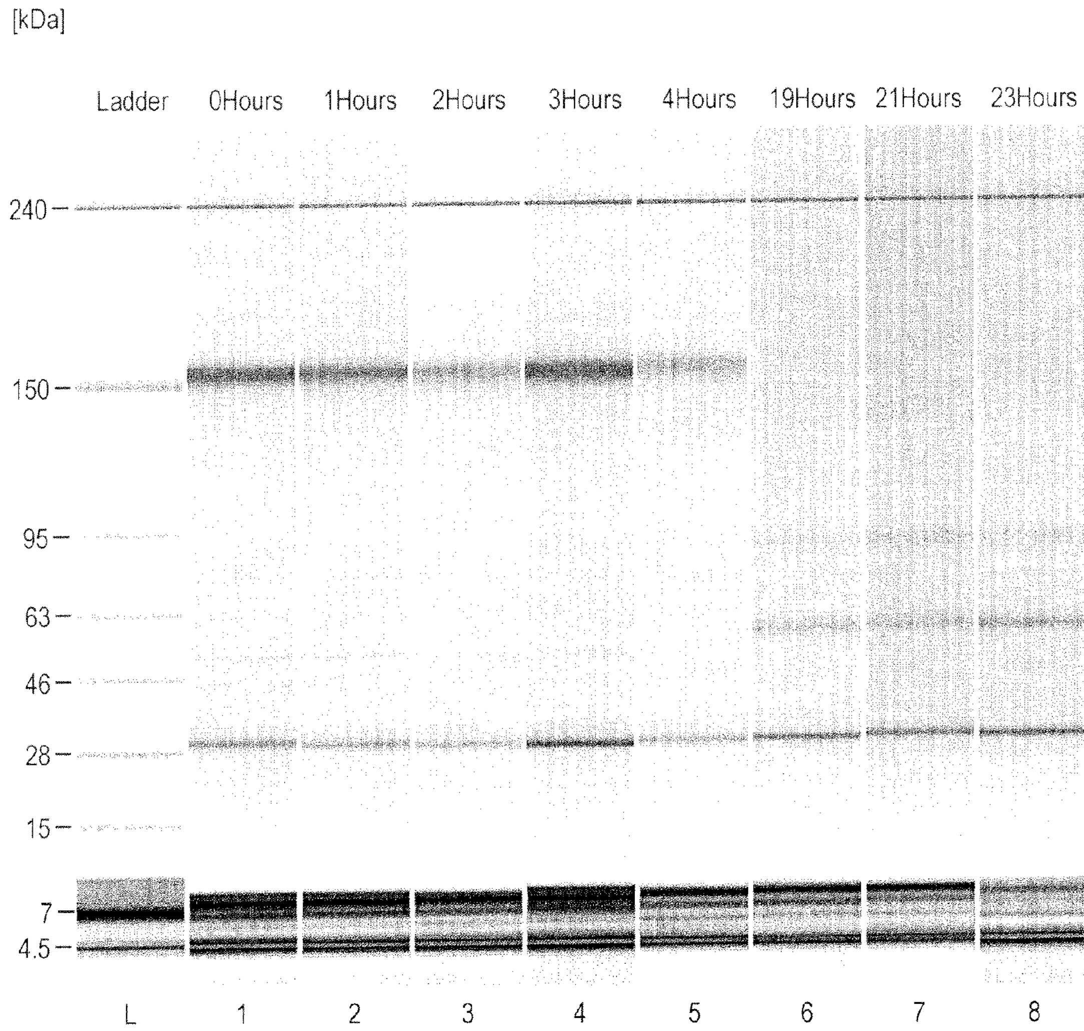
Inhibition of Ocrelizumab Reduction In HCCF by Aurothiomalate

FIG. 12



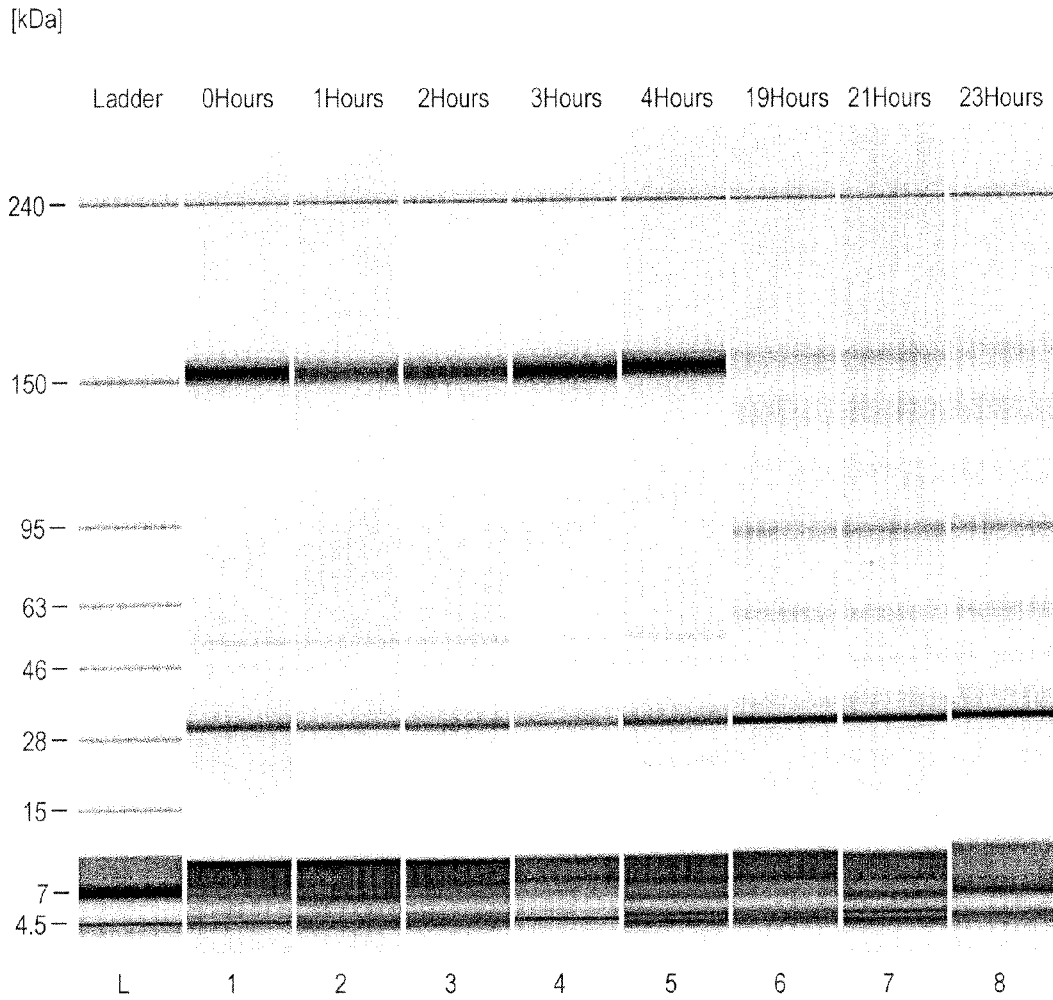
Losing Reduction Activity in HCCF

FIG. 13



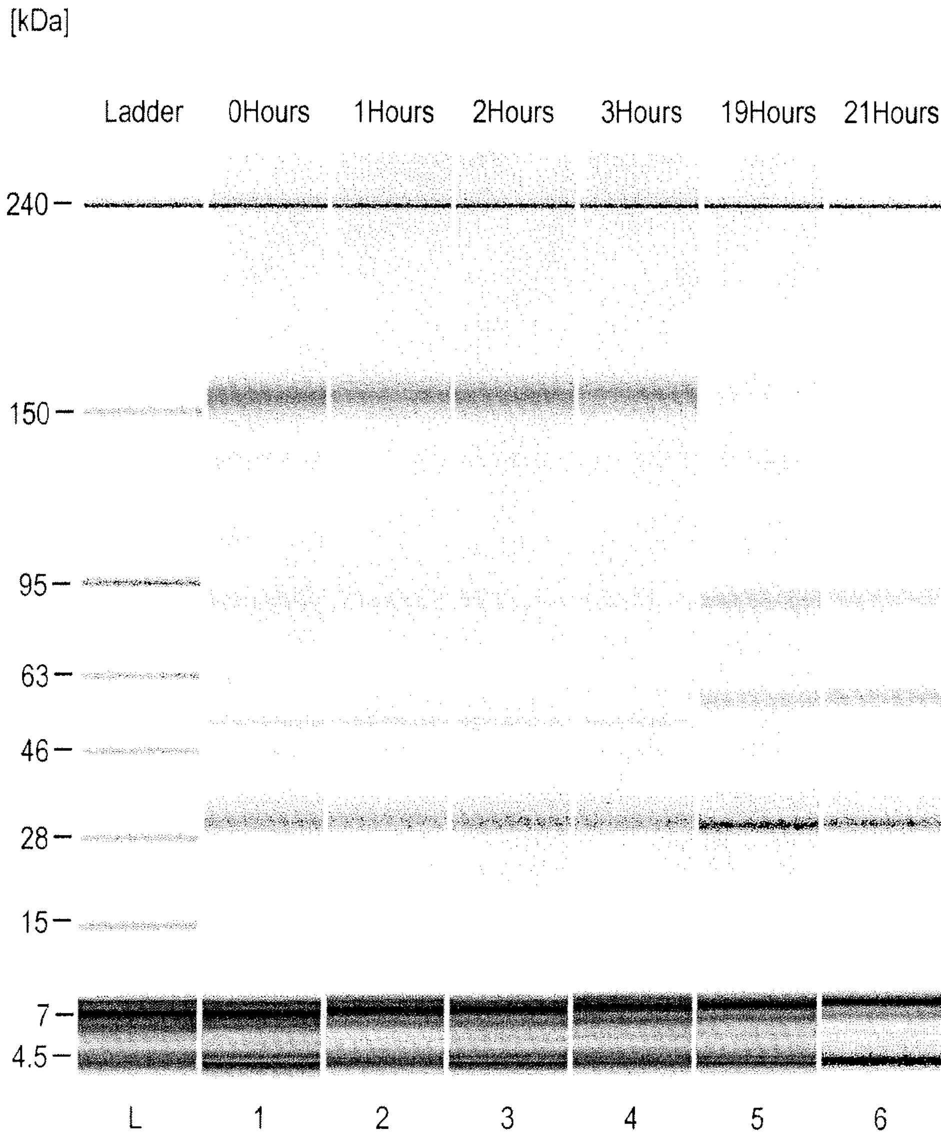
The Lost Reduction Activity in HCCF Restored by Addition of NADPH

FIG. 14



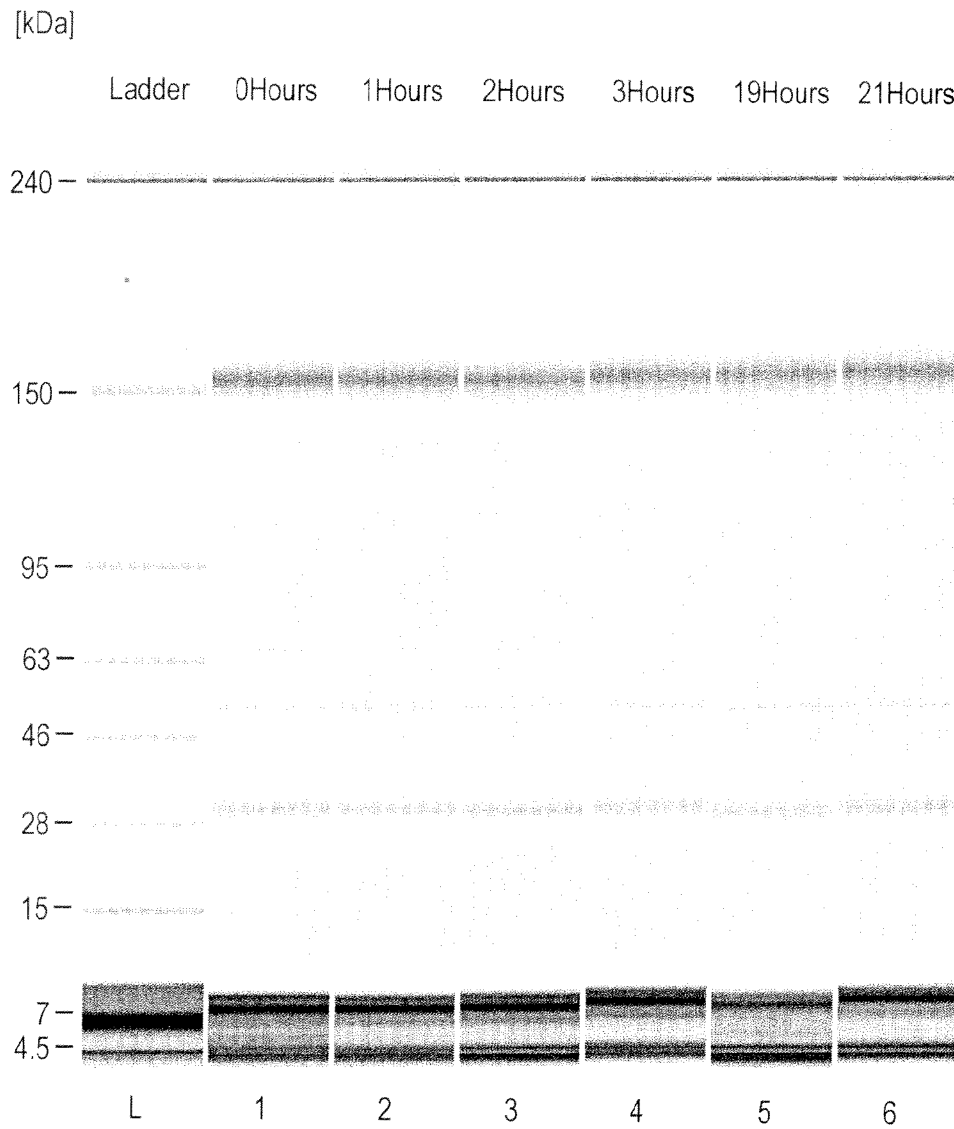
The Lost Reduction Activity in HCCF Restored by Addition of Glucose-6-Phosphate

FIG. 15



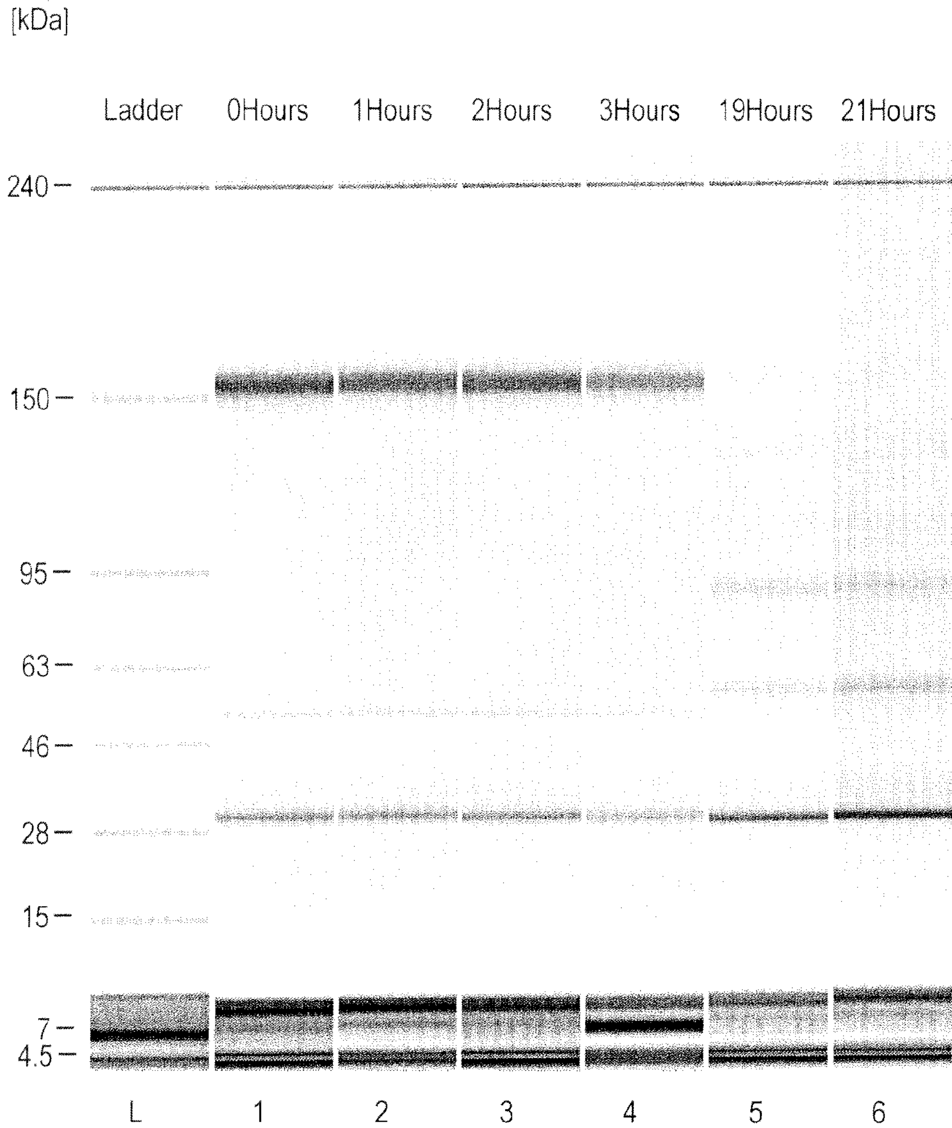
Ocrelizumab Reduction

FIG. 16



EDTA Inhibits Ocrelizumab Reduction

FIG. 17



The Lost Reduction Activity in Run 8 HCCF Restored by Addition of Glucose-6-Phosphate but No Inhibition of Reduction by EDTA

FIG. 18

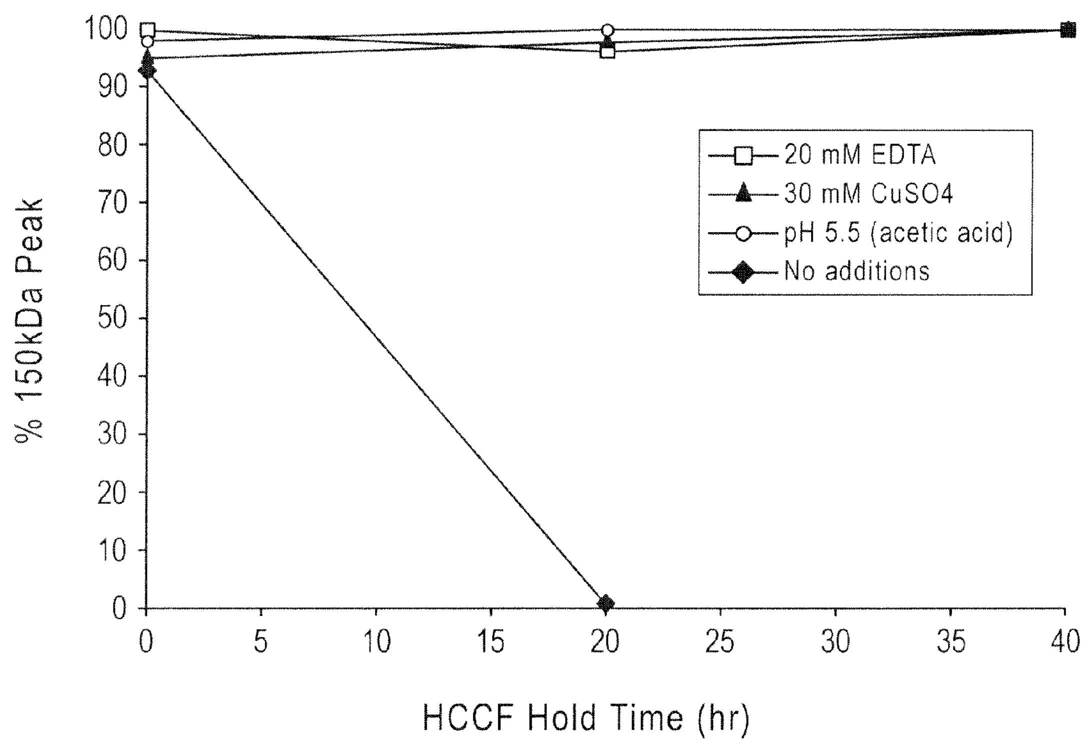


FIG. 19

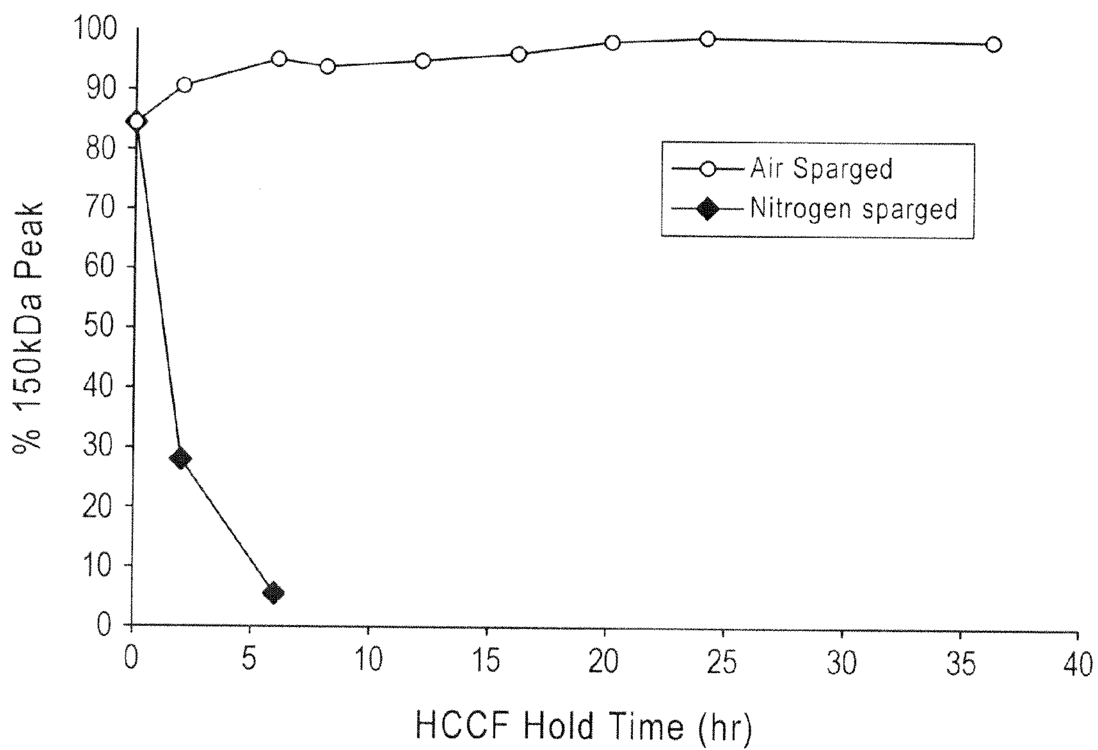


FIG. 20

Light Chain

```
1              15               30             45
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPK

46             60               75             90
LLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQ

91             105
HYTTPPTFGQGTKVEIK
```

FIG. 21

Heavy Chain

```
1              15               30             45
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGL

46             60               75             90
EWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAED

91             105              120
TAVYYCSRWGGDGFYAMDYWGQGTLVTVSS
```

FIG. 22

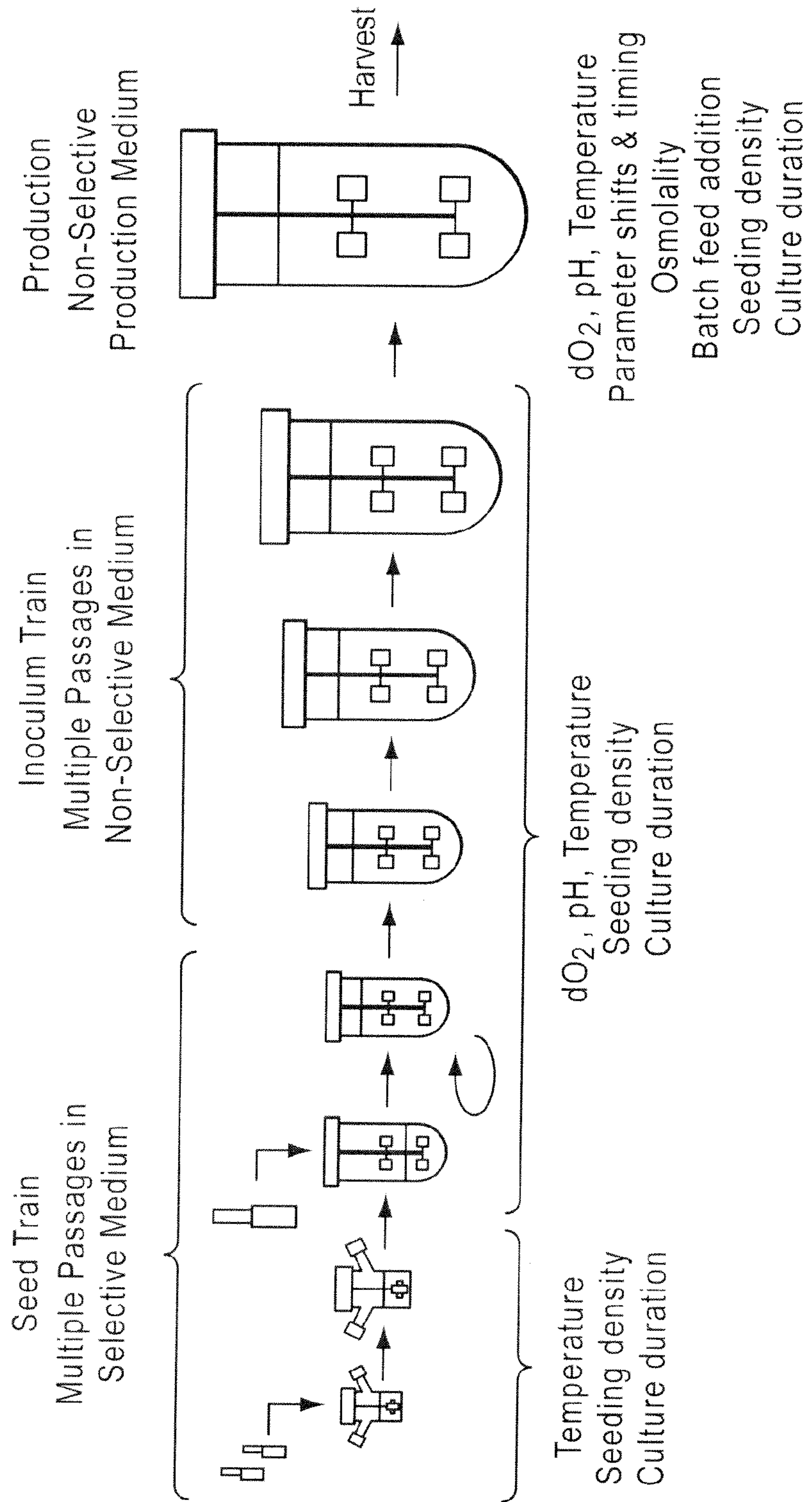


FIG. 23

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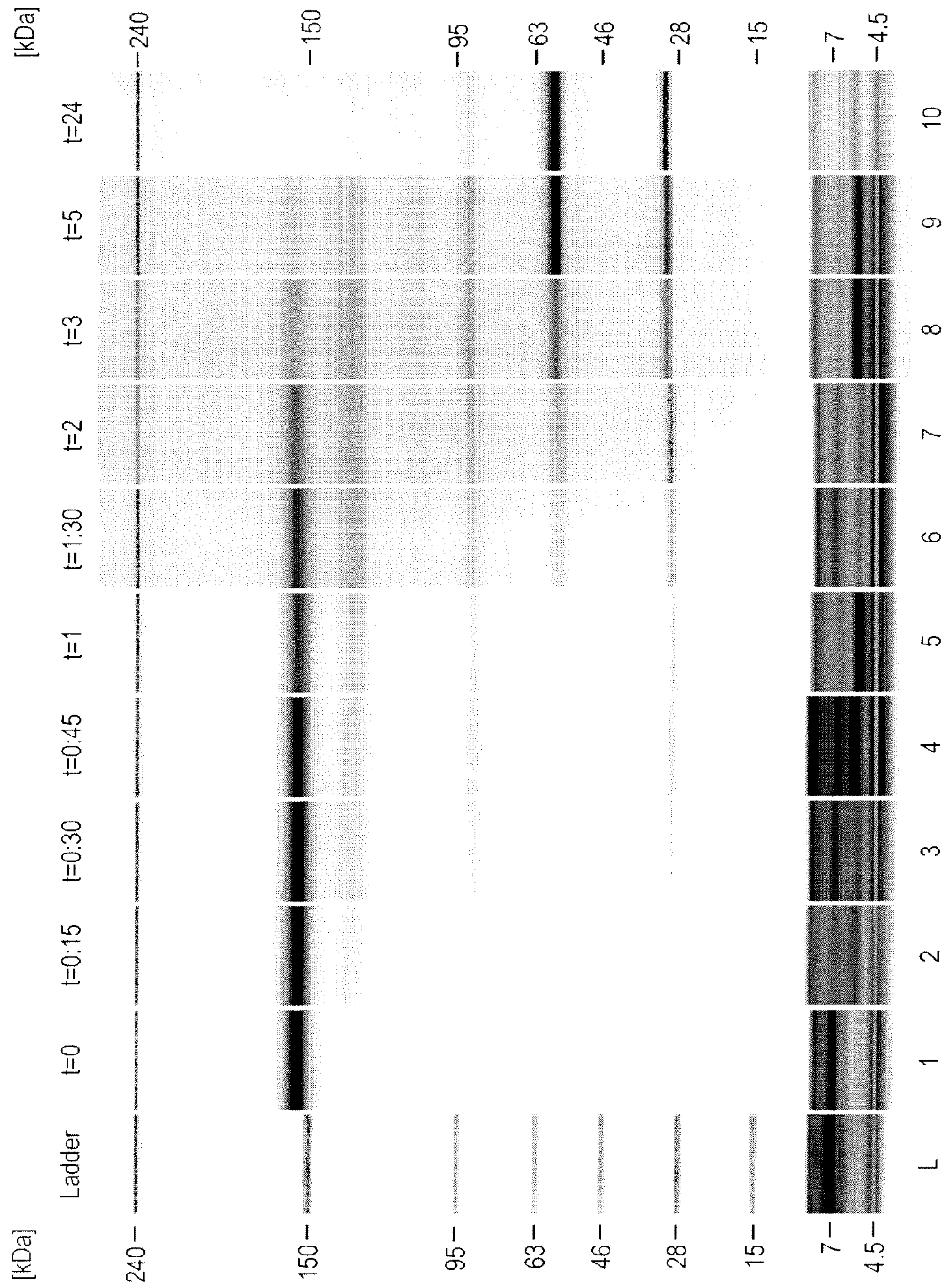

FIG. 24

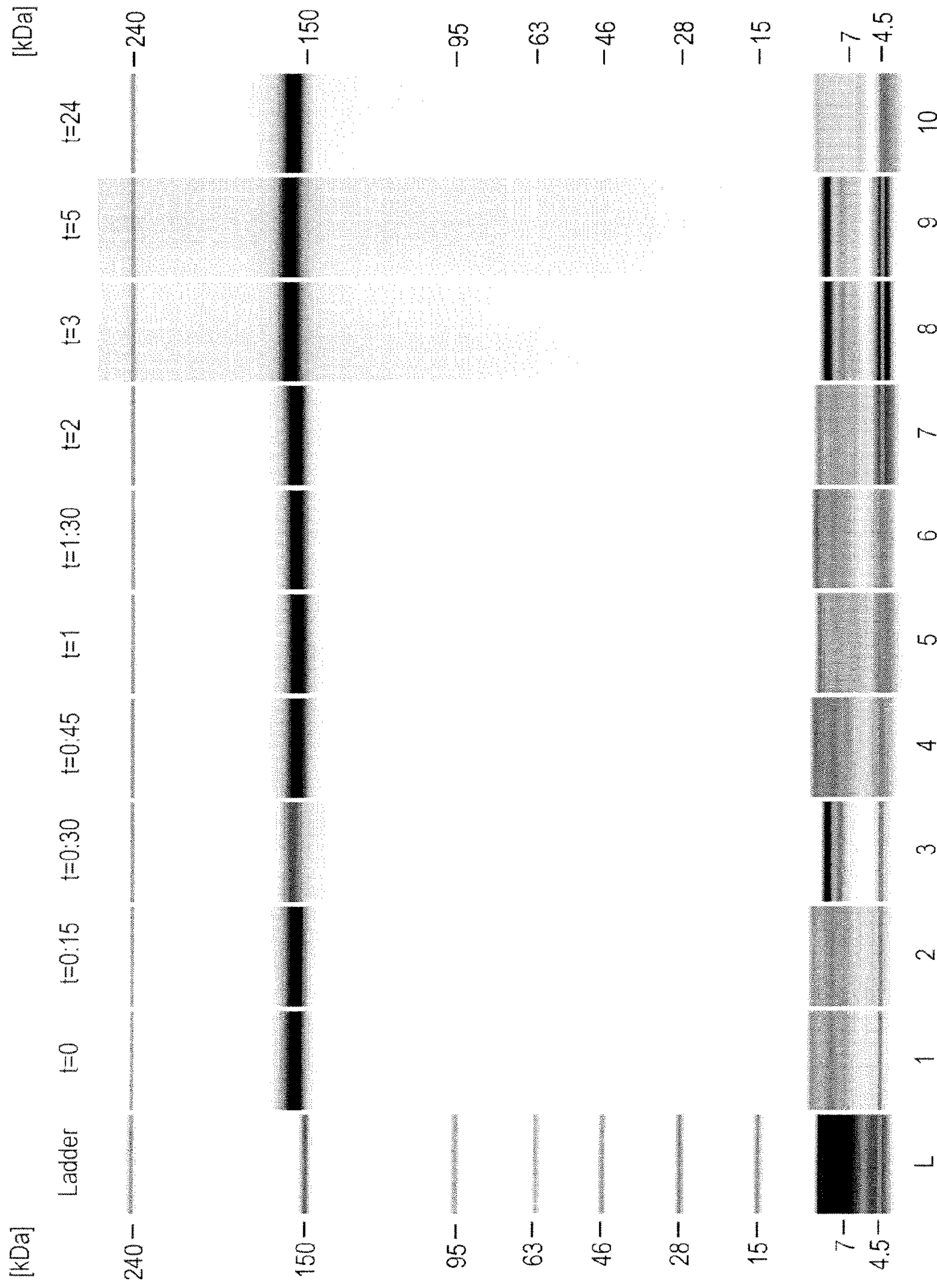

FIG. 25

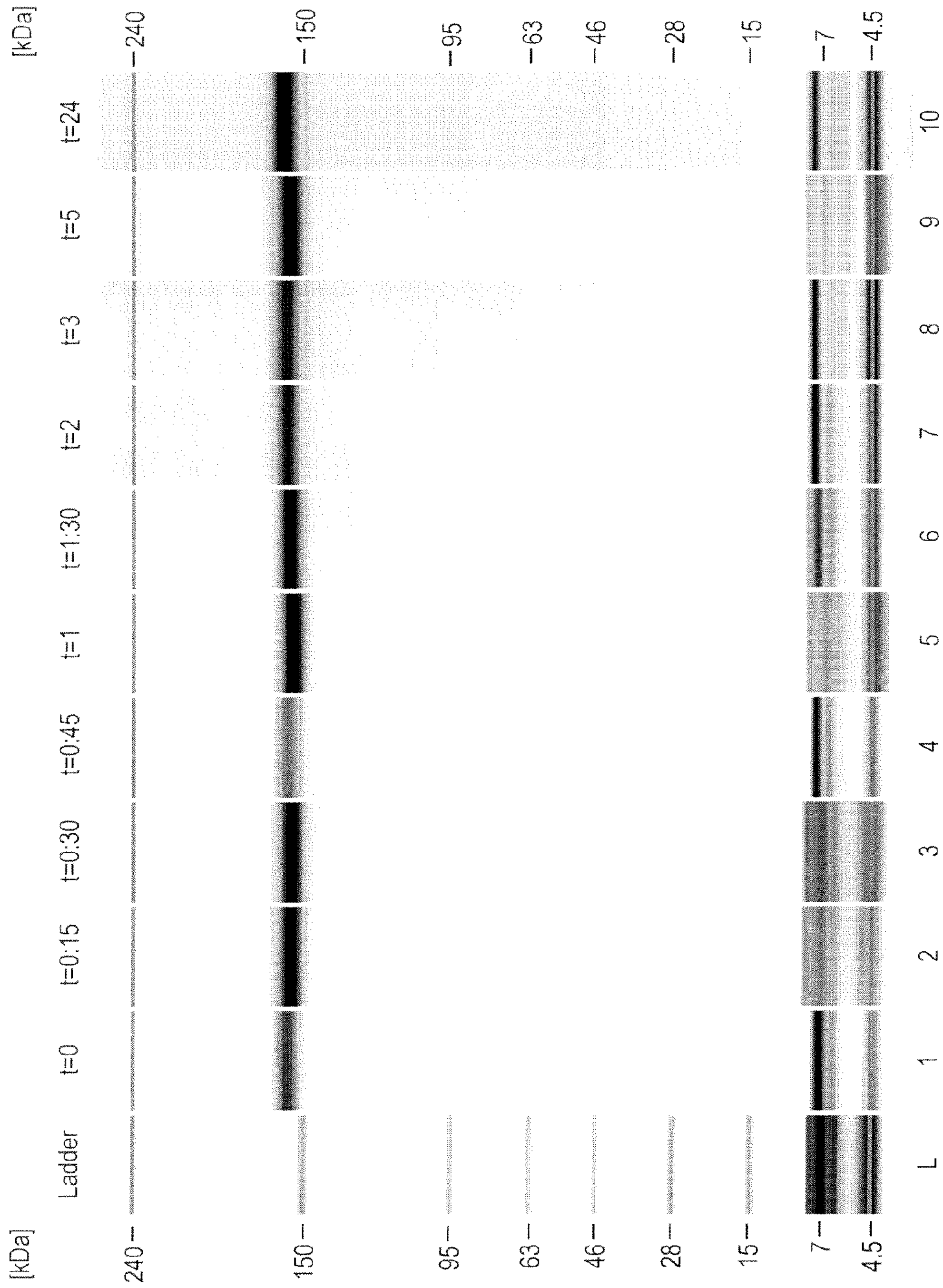

FIG. 26

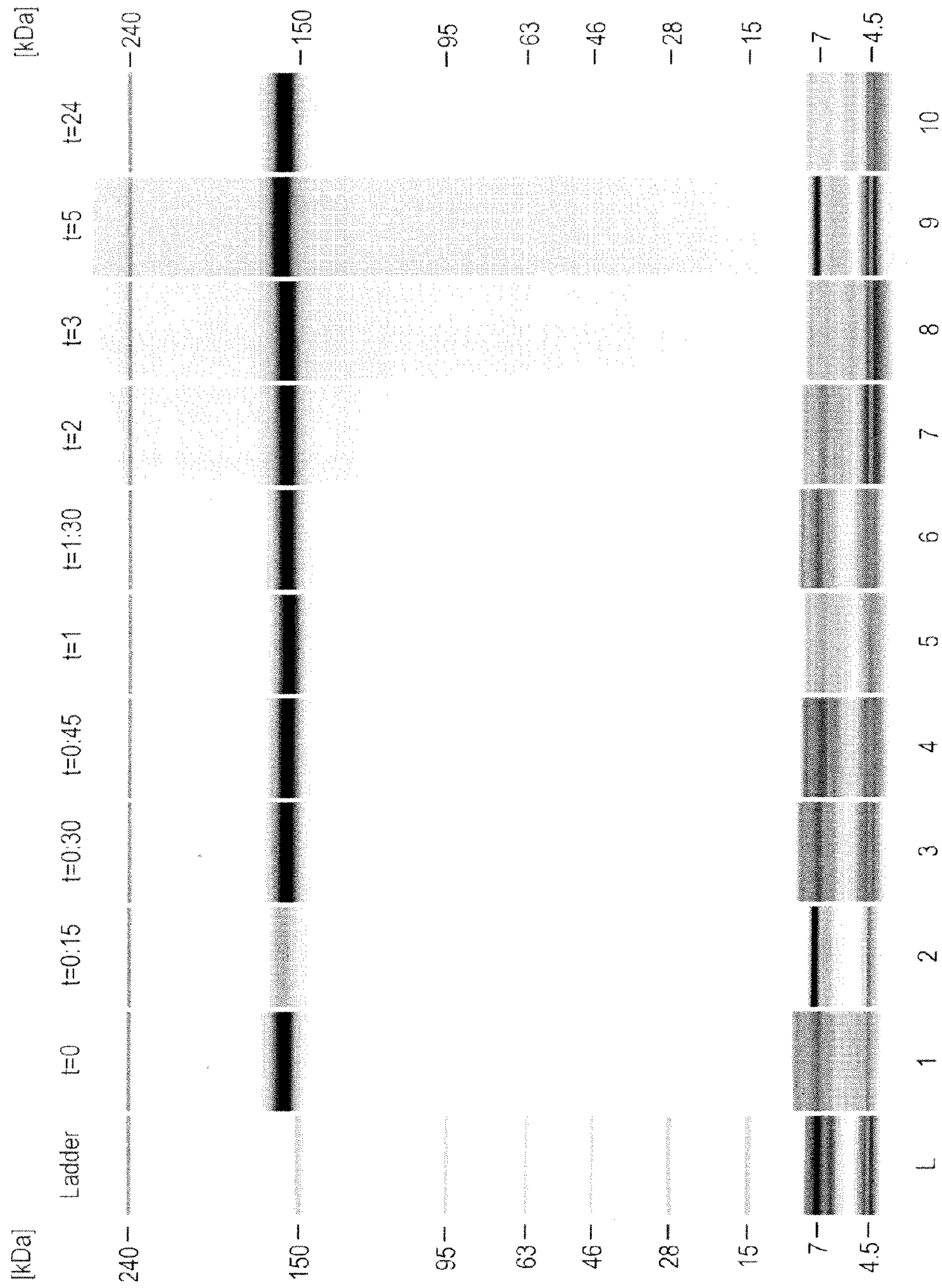

FIG. 27

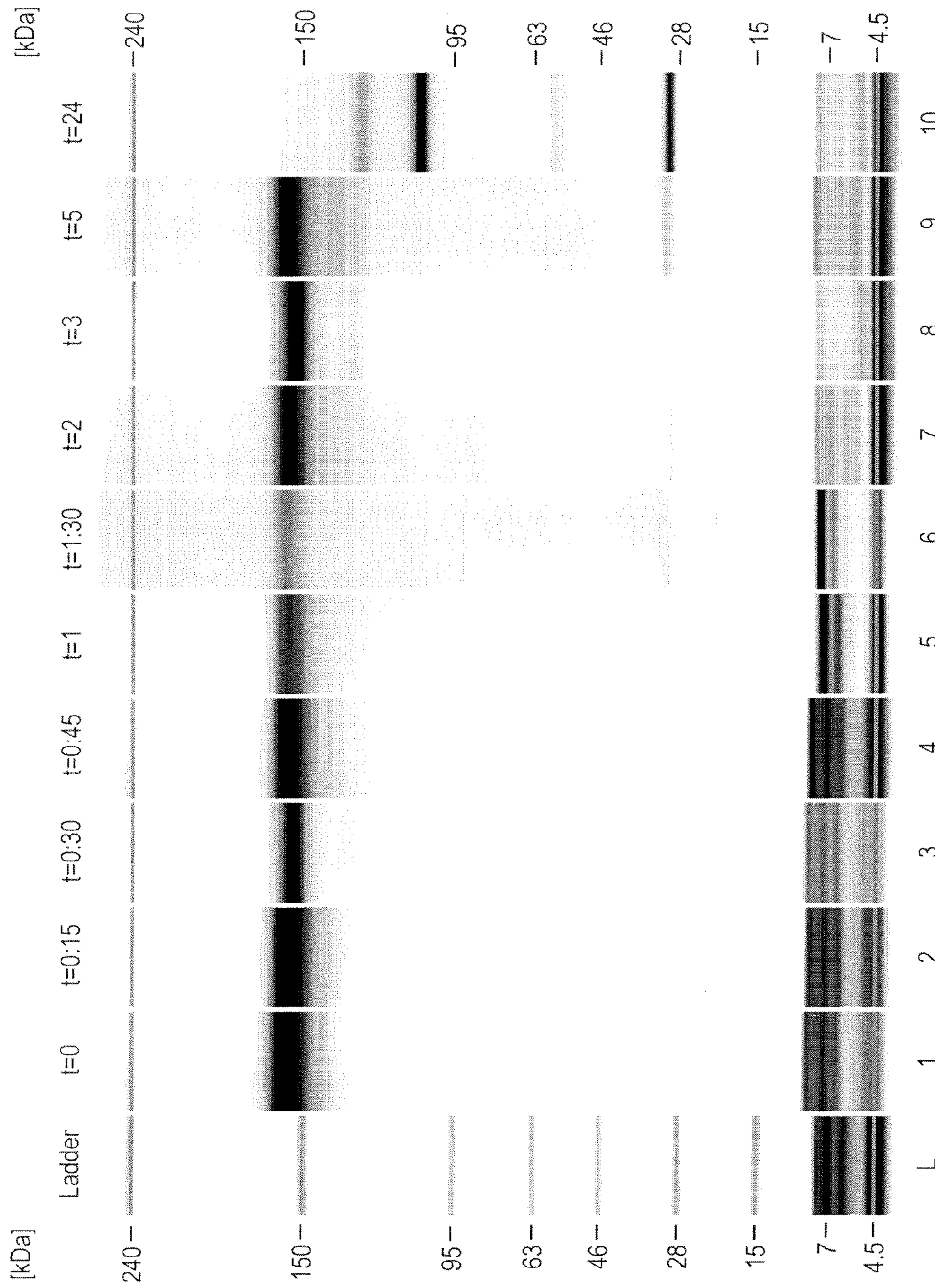

FIG. 28

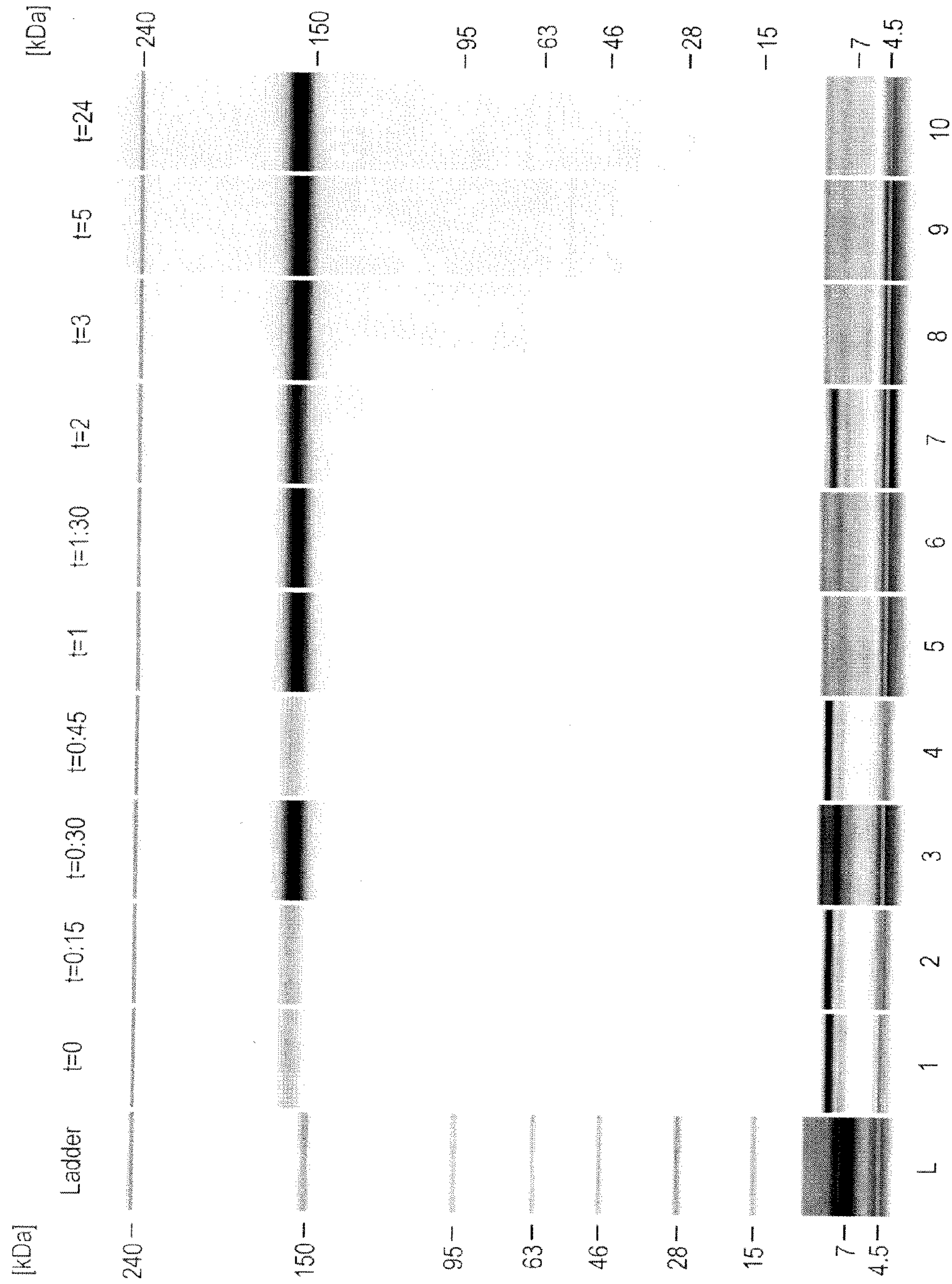

FIG. 29

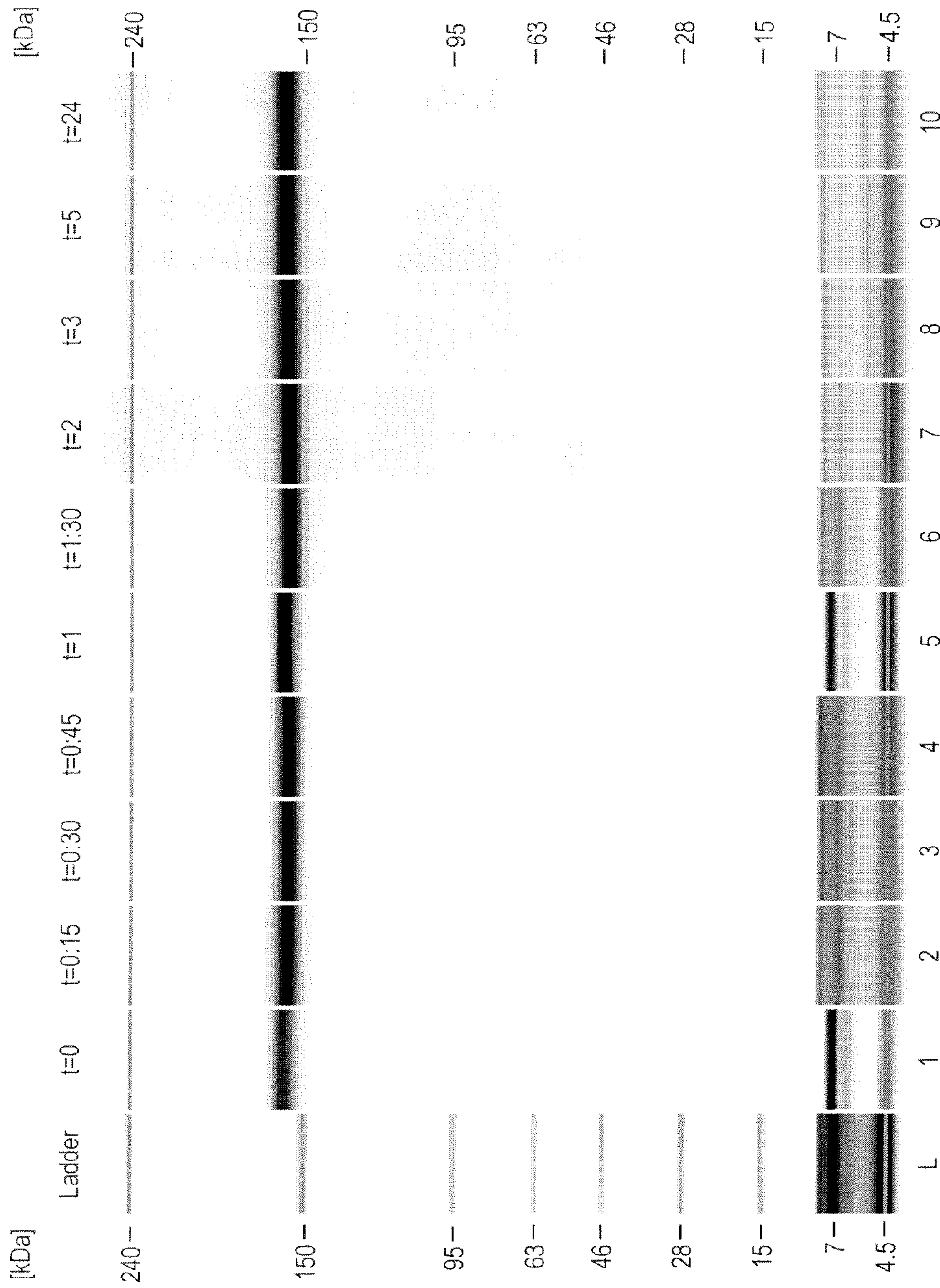

FIG. 30

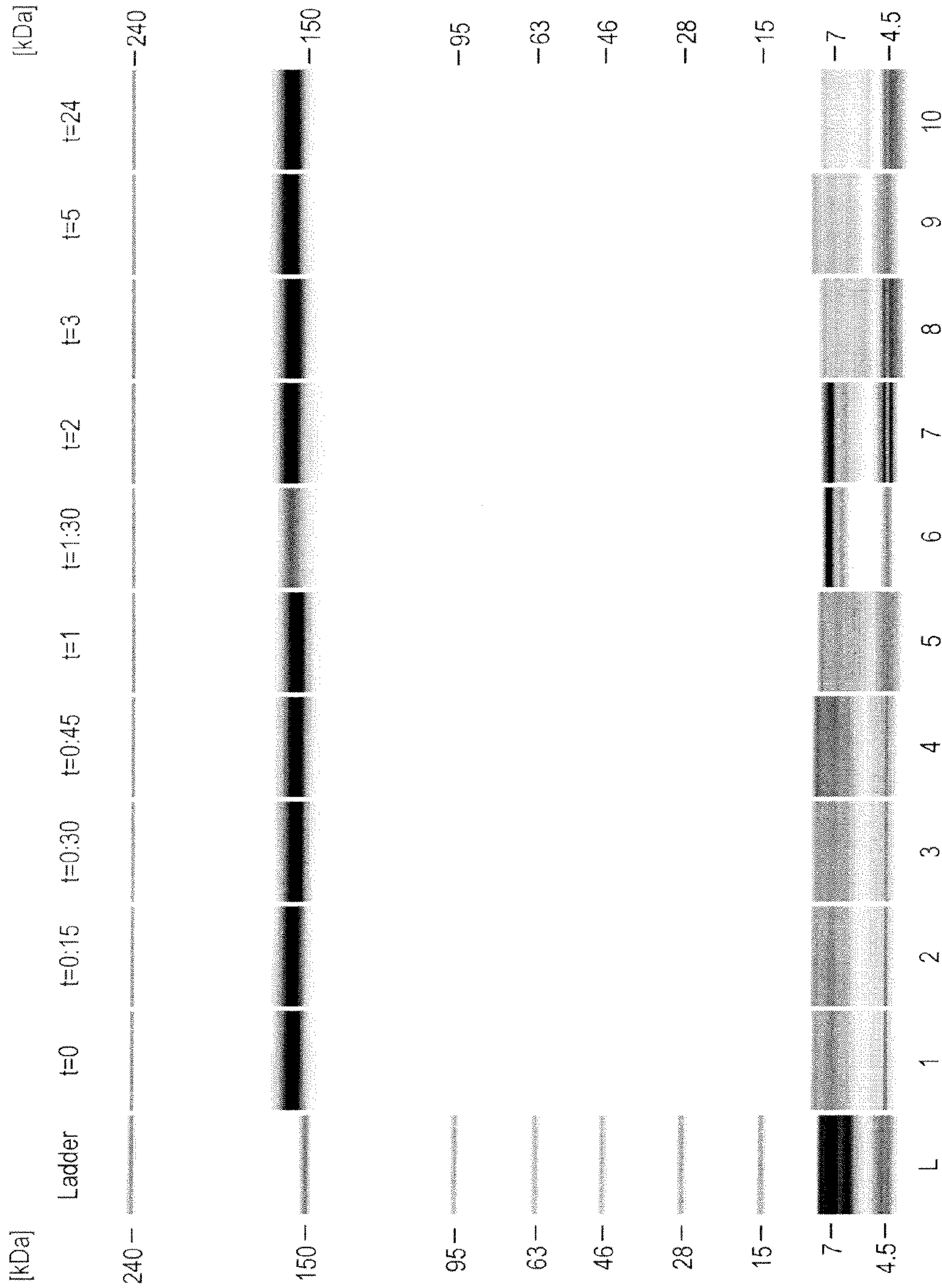

FIG. 31

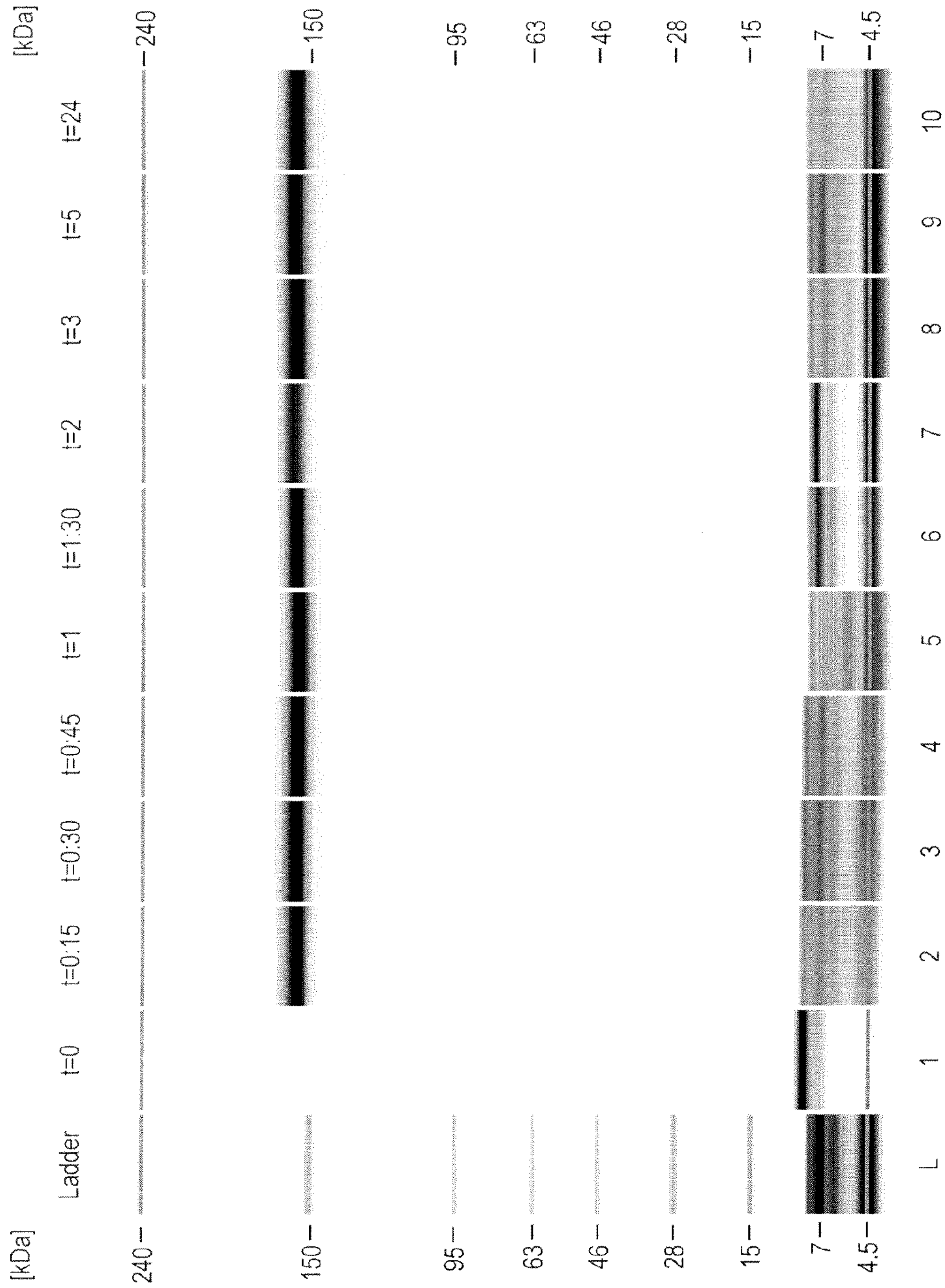

FIG. 32

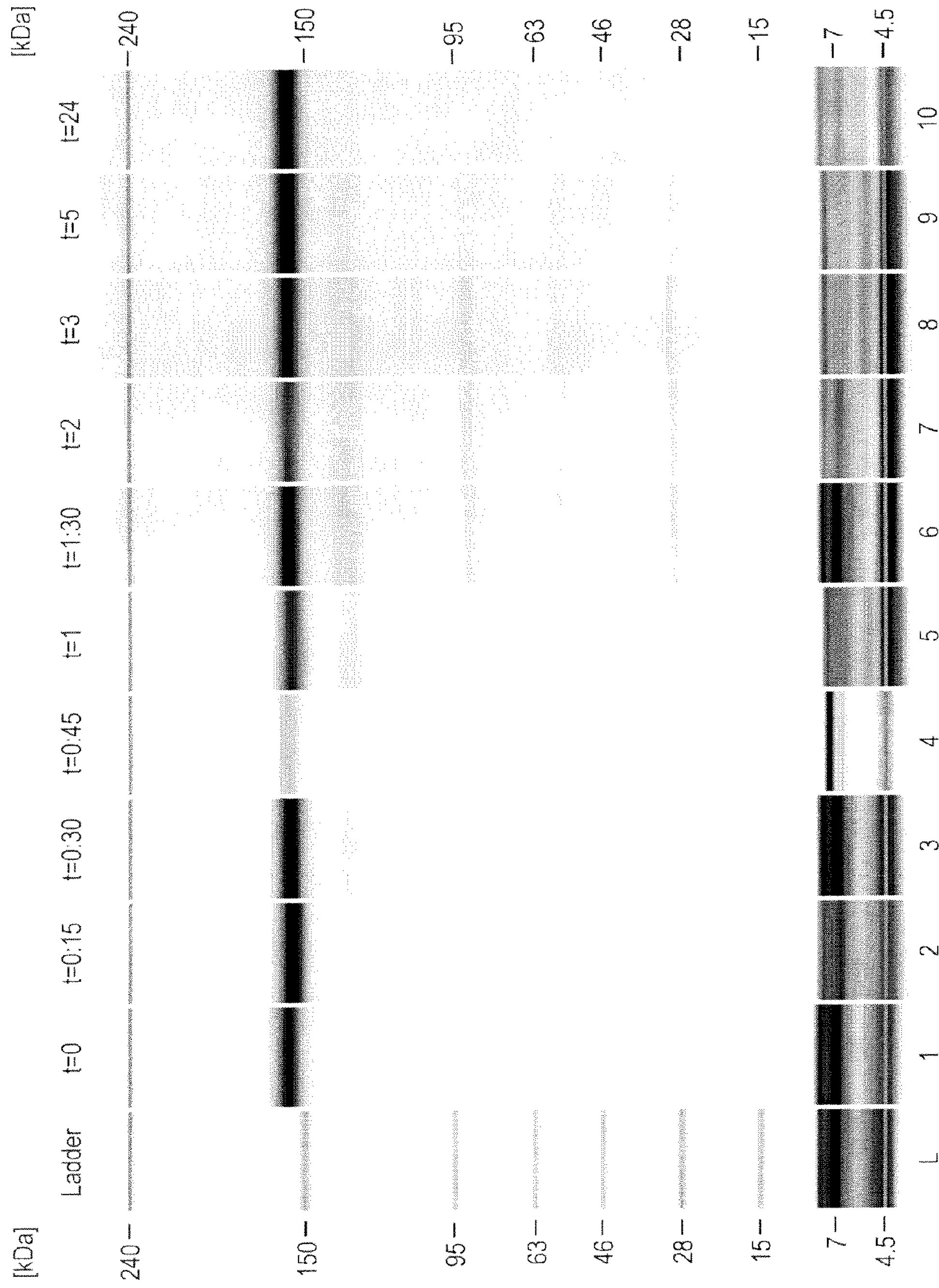

FIG. 33

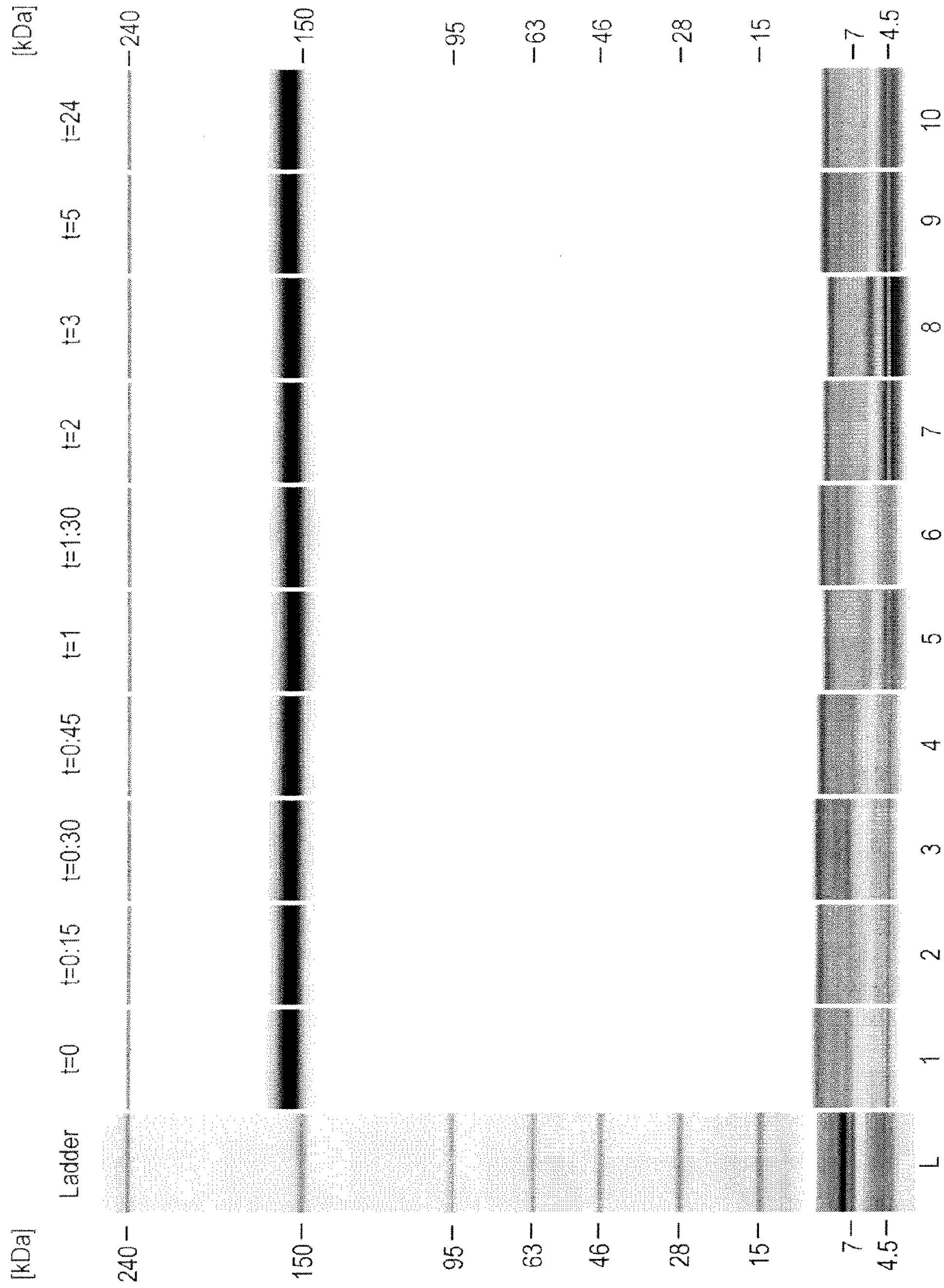

FIG. 34

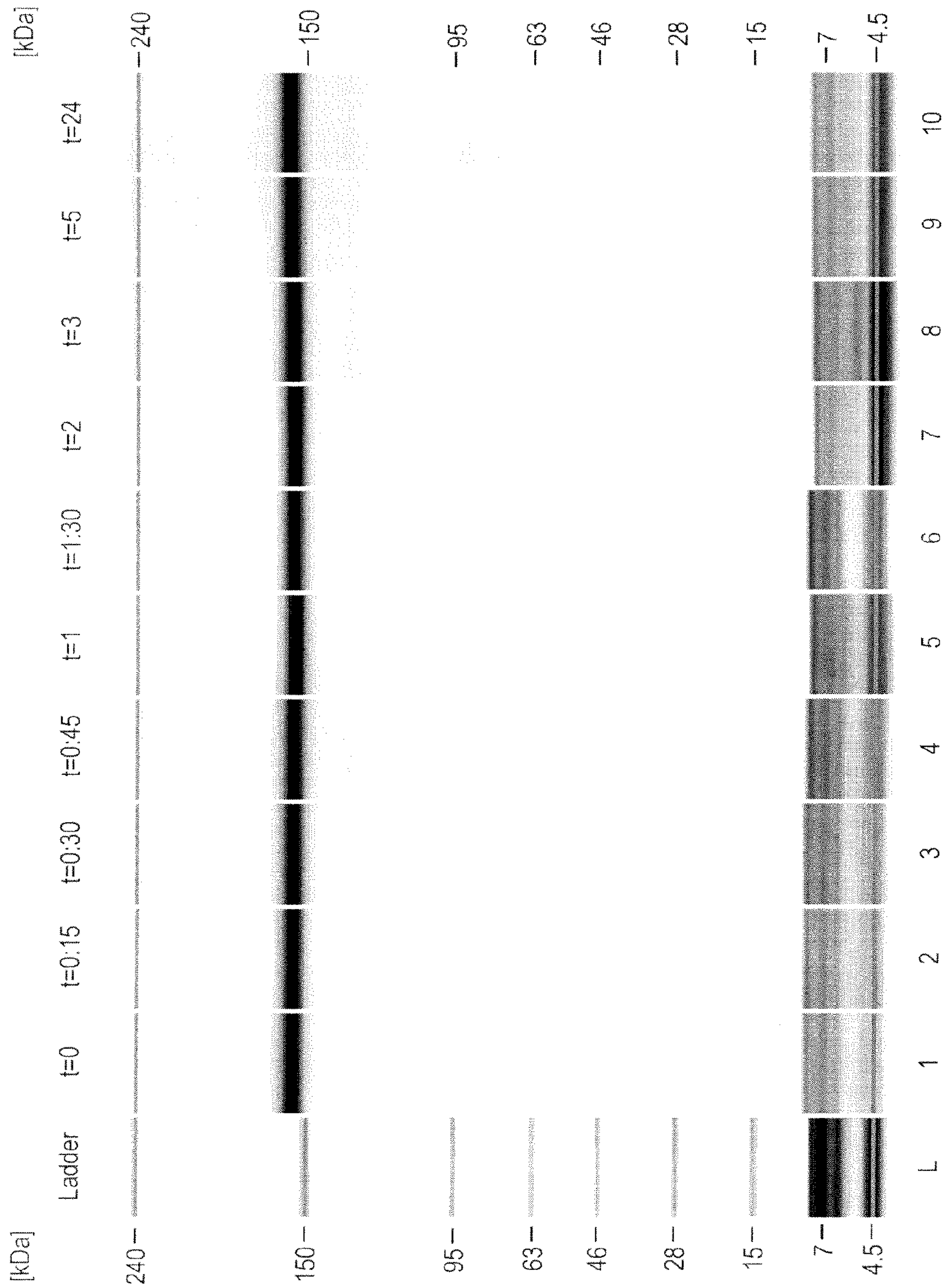

FIG. 35

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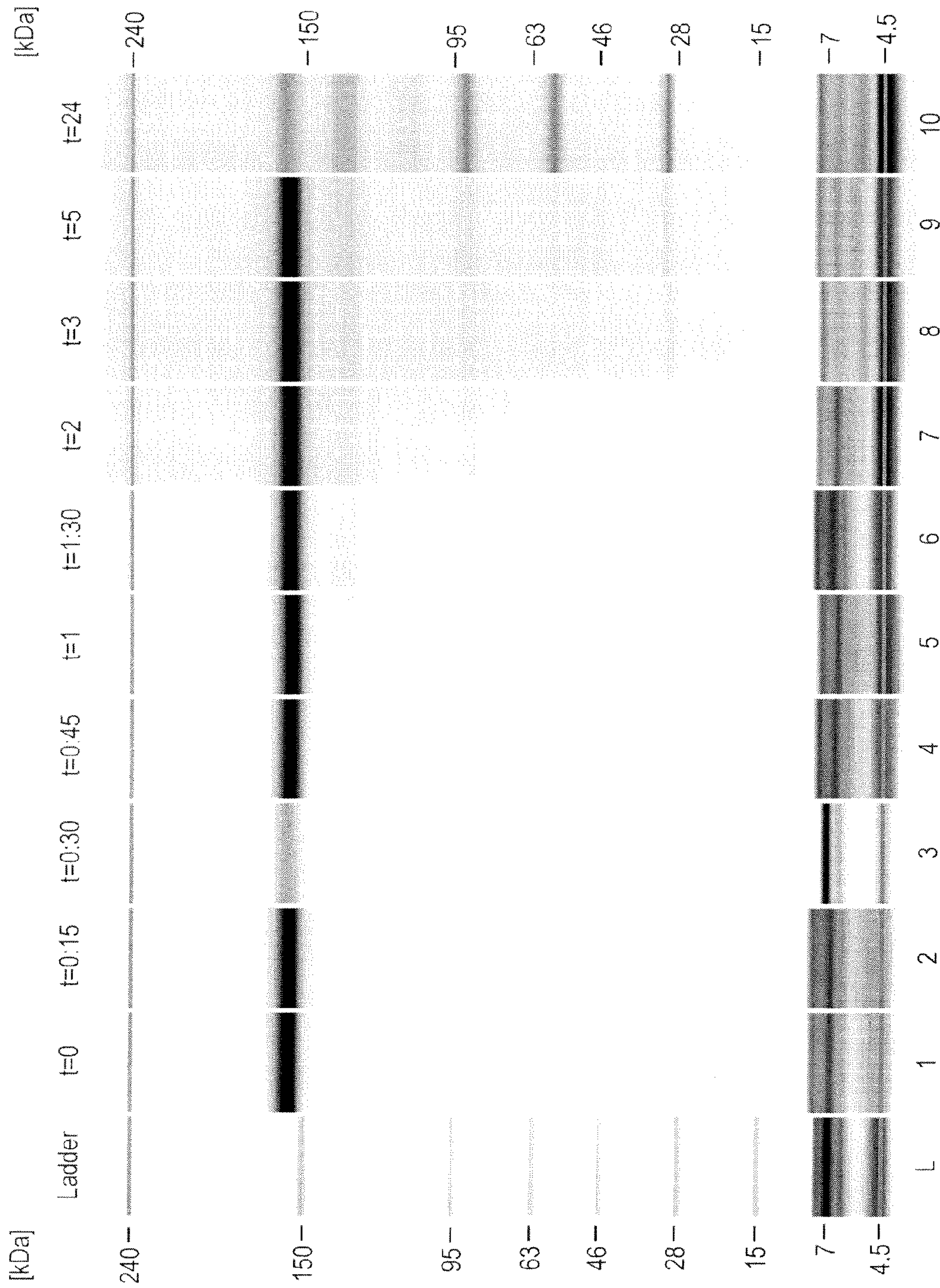

FIG. 36

Case 1:18-cv-00095-CFC Document 1-3 Filed 01/12/18 Page 281 of 681 PageID #: 1150

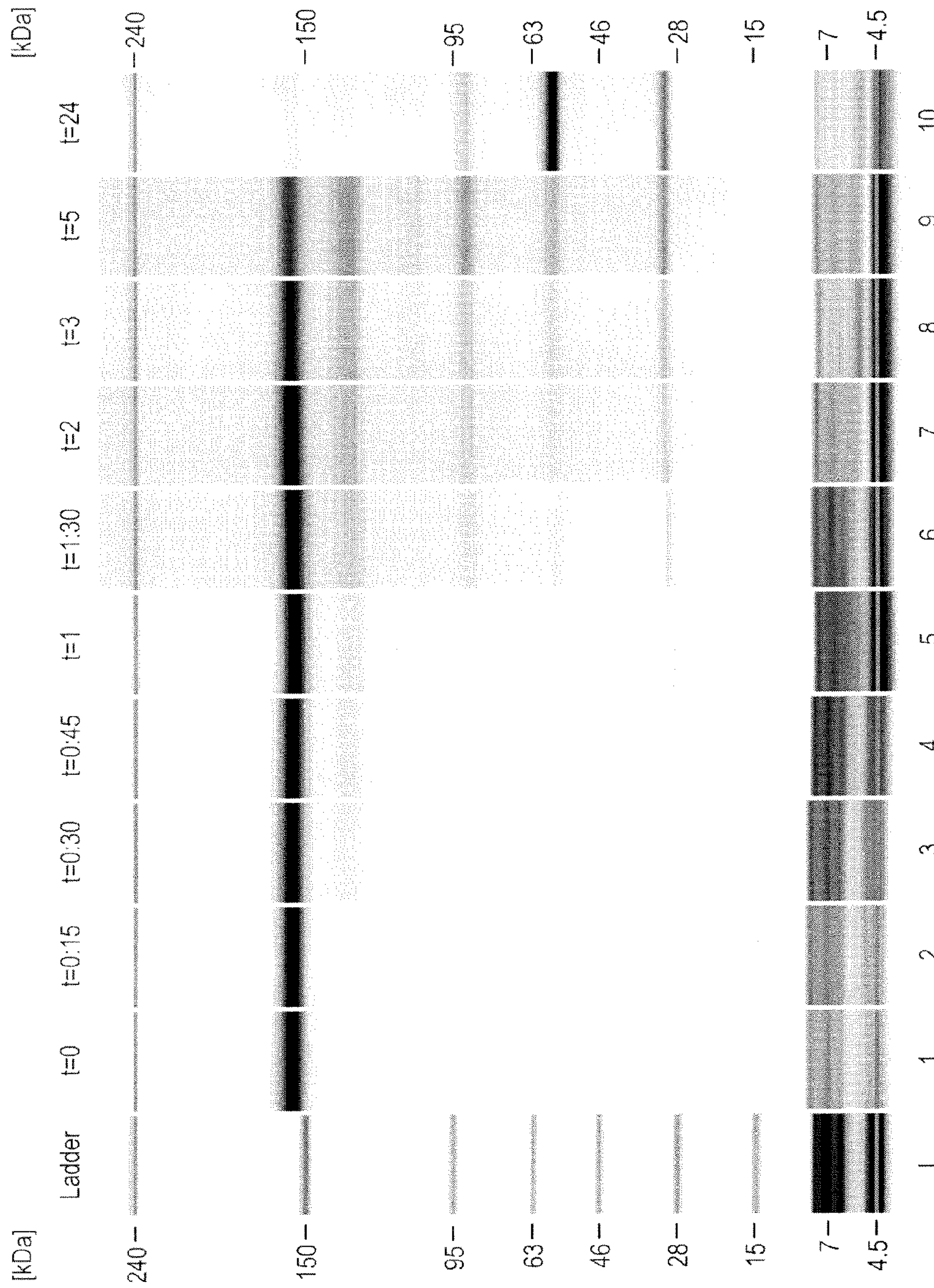

FIG. 37

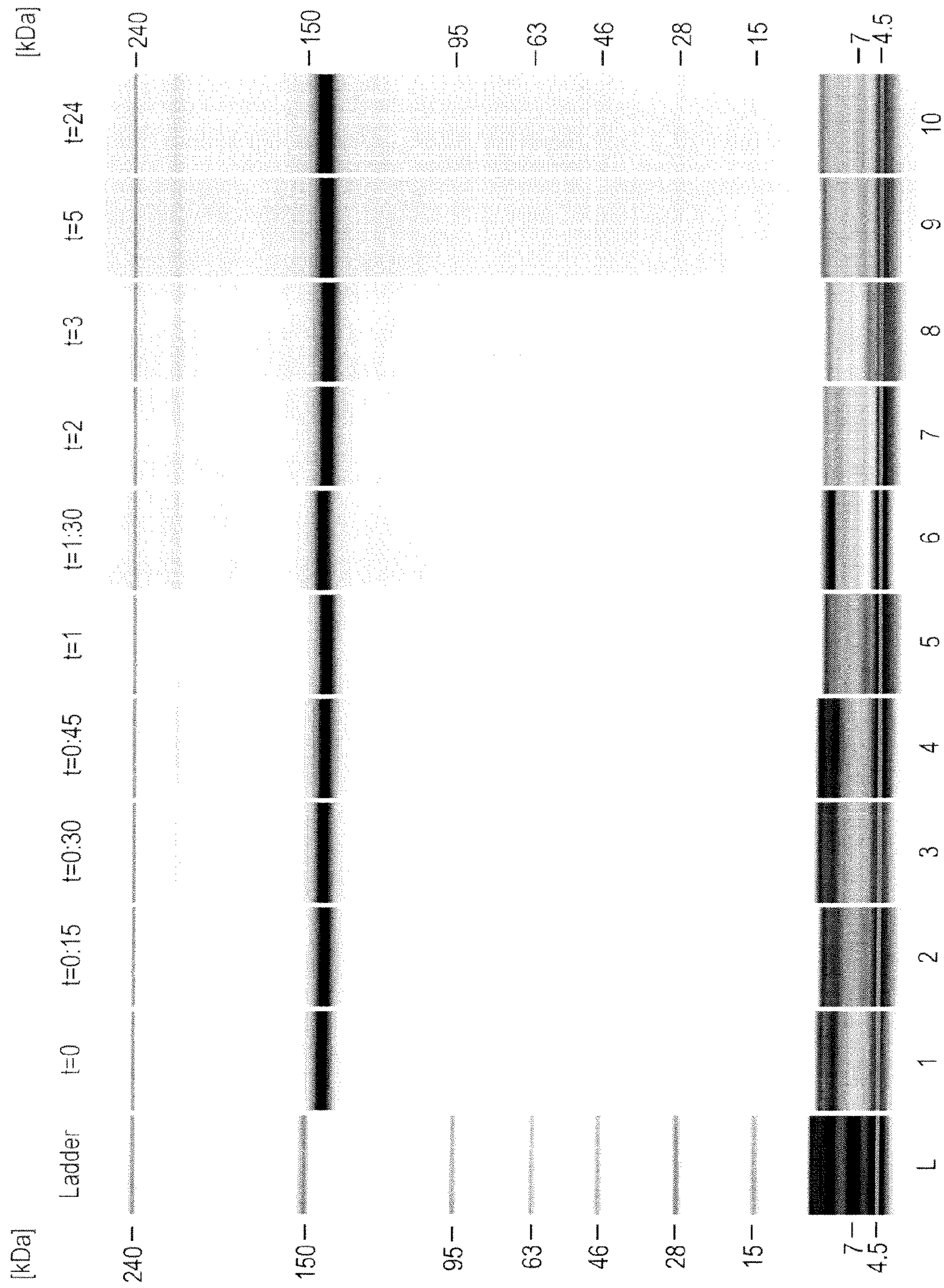

FIG. 38

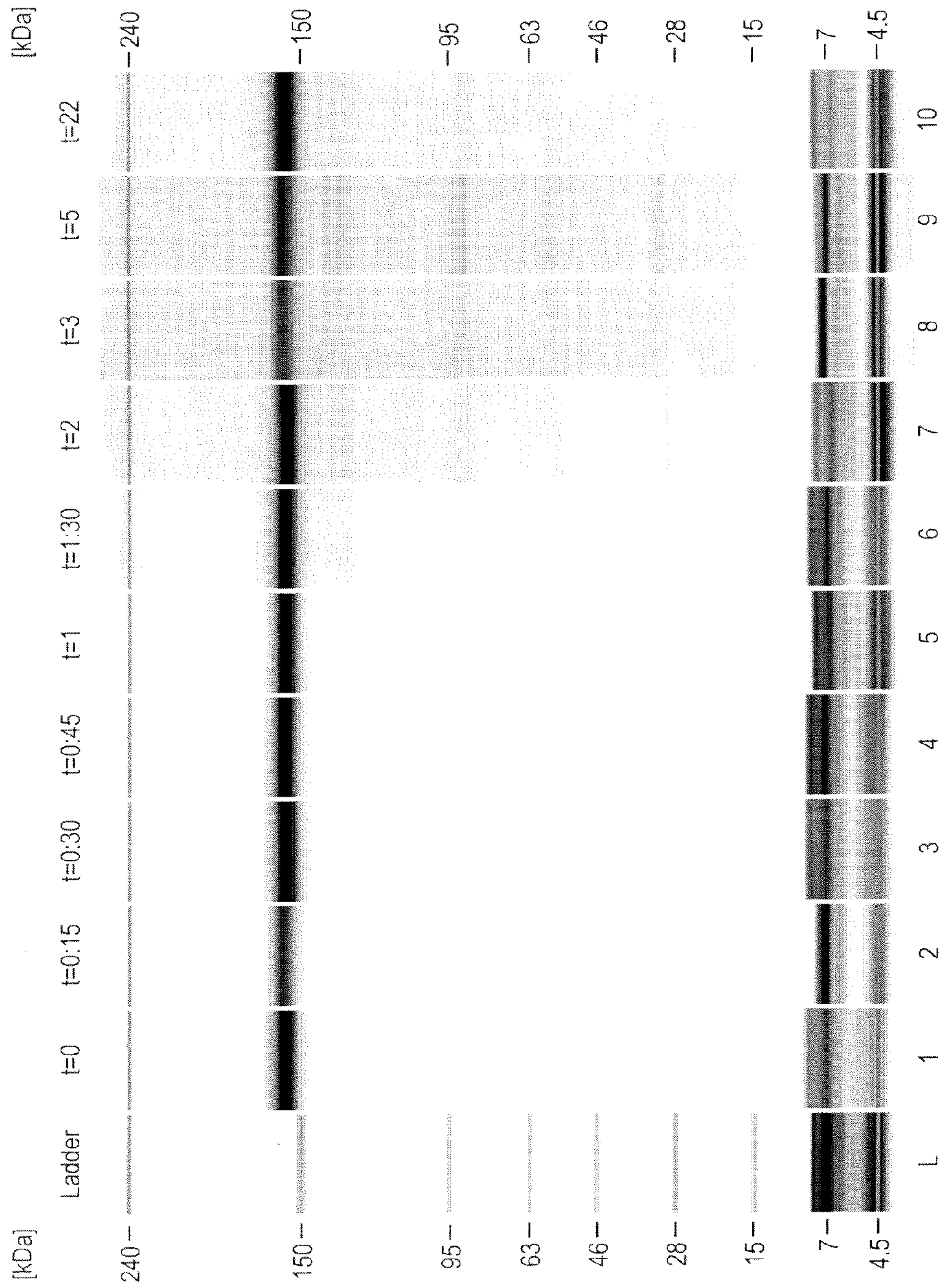

FIG. 39

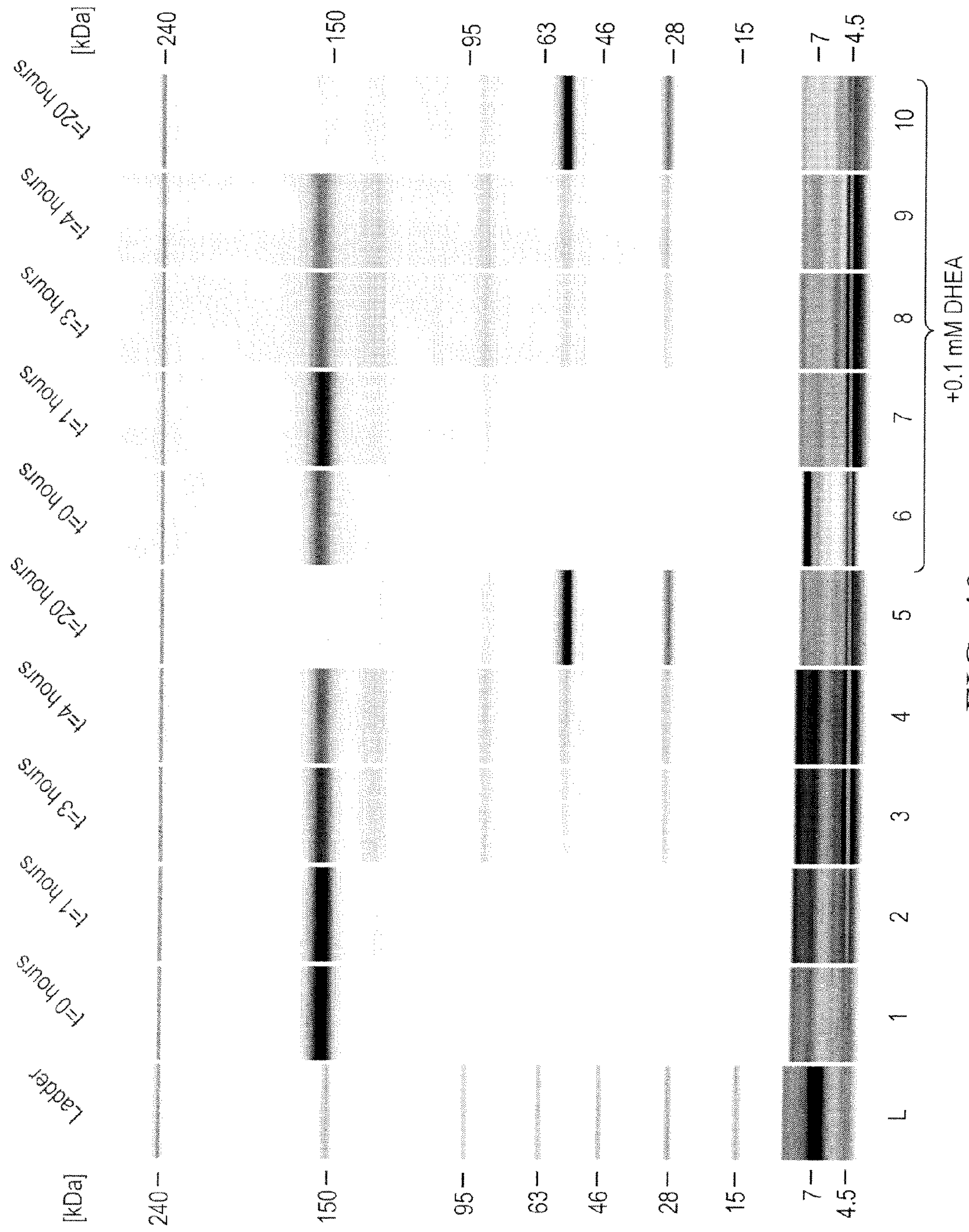

FIG. 40

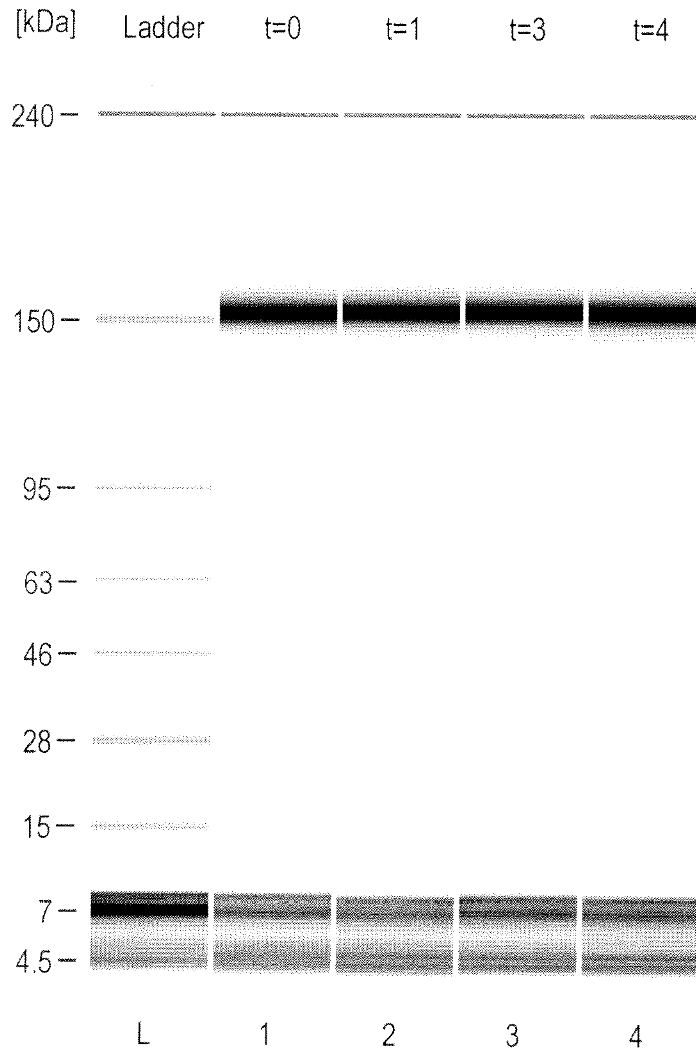

FIG. 41

PREVENTION OF DISULFIDE BOND REDUCTION DURING RECOMBINANT PRODUCTION OF POLYPEPTIDES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 12/217,745, filed Jul. 8, 2008, which is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional Application No. 60/948,677 filed Jul. 9, 2007, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The invention concerns methods and means for preventing the reduction of disulfide bonds during the recombinant production of disulfide-containing polypeptides. In particular, the invention concerns the prevention of disulfide bond reduction during harvesting of disulfide-containing polypeptides, including antibodies, from recombinant host cell cultures.

BACKGROUND OF THE INVENTION

In the biotechnology industry, pharmaceutical applications require a variety of proteins produced using recombinant DNA techniques. Generally, recombinant proteins are produced by cell culture, using either eukaryotic cells, such as mammalian cells, or prokaryotic cells, such as bacterial cells, engineered to produce the protein of interest by insertion of a recombinant plasmid containing the nucleic acid encoding the desired protein. For a protein to remain biologically active, the conformation of the protein, including its tertiary structure, must be maintained during its purification and isolation, and the protein's multiple functional groups must be protected from degradation.

Mammalian cells have become the dominant system for the production of mammalian proteins for clinical applications, primarily due to their ability to produce properly folded and assembled heterologous proteins, and their capacity for post-translational modifications. Chinese hamster ovary (CHO) cells, and cell lines obtained from various other mammalian sources, such as, for example, mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) and human retinal cells, such as the PER.C6® cell line isolated from a human retinal cell, which provides human glycosylation characteristics, and is able to naturally produce antibodies that match human physiology, have been approved by regulatory agencies for the production of biopharmaceutical products.

Usually, to begin the production cycle, a small number of transformed recombinant host cells are allowed to grow in culture for several days (see, e.g., FIG. **23**). Once the cells have undergone several rounds of replication, they are transferred to a larger container where they are prepared to undergo fermentation. The media in which the cells are grown and the levels of oxygen, nitrogen and carbon dioxide that exist during the production cycle may have a significant impact on the production process. Growth parameters are determined specifically for each cell line and these parameters are measured frequently to assure optimal growth and production conditions.

When the cells grow to sufficient numbers, they are transferred to large-scale production tanks and grown for a longer period of time. At this point in the process, the recombinant protein can be harvested. Typically, the cells are engineered to

secrete the polypeptide into the cell culture media, so the first step in the purification process is to separate the cells from the media. Typically, harvesting includes centrifugation and filtration to produce a Harvested Cell Culture Fluid (HCCF). The media is then subjected to several additional purification steps that remove any cellular debris, unwanted proteins, salts, minerals or other undesirable elements. At the end of the purification process, the recombinant protein is highly pure and is suitable for human therapeutic use.

Although this process has been the subject of much study and improvements over the past several decades, the production of recombinant proteins is still not without difficulties. Thus, for example, during the recombinant production of polypeptides comprising disulfide bonds, especially multi-chain polypeptides comprising inter-chain disulfide bonds such as antibodies, it is essential to protect and retain the disulfide bonds throughout the manufacturing, recovery and purification process, in order to produce properly folded polypeptides with the requisite biological activity.

SUMMARY OF THE INVENTION

The instant invention generally relates to a method for preventing reduction of a disulfide bond in a polypeptide expressed in a recombinant host cell, comprising supplementing the pre-harvest or harvested culture fluid of the recombinant host cell with an inhibitor of thioredoxin or a thioredoxin-like protein.

In one embodiment, the thioredoxin inhibitor is added to the pre-harvest culture fluid.

In another embodiment, the thioredoxin inhibitor is added to the harvested culture fluid.

In a further embodiment, the thioredoxin inhibitor is a direct inhibitor of thioredoxin.

In all embodiments, the thioredoxin inhibitor may, for example, be an alkyl-2-imidazolyl disulfide or a naphthoquinone spiroketal derivative.

In a further embodiment, the thioredoxin inhibitor is a specific inhibitor of thioredoxin reductase.

In a still further embodiment, the thioredoxin inhibitor is a gold complex, where the gold complex may, for example, be aurothioglucose (ATG) or aurothiomalate (ATM). While the effective inhibitory concentration may vary, it typically is between about 0.1 mM and 1 mM. Similarly, the minimum effective inhibitory concentration varies depending on the nature of the polypeptide and overall circumstances, and is typically reached when the ATG or ATG concentration is at least about four-times of thioreduxin concentration in the pre-harvest or harvested culture fluid.

In another embodiment of this aspect of the invention, the thioredoxin inhibitor is a metal ion, where the metal ion, without limitation, may be selected from the group consisting of Hg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+}. When the metal ion is added in the form of cupric sulfate, the effective inhibitory concentration generally is between about 5 μM and about 100 μM, or between about 10 μM and about 80 μM, or between about 15 μM and about 50 μM. The minimum inhibitory concentration of cupric sulfate also varies, but typically is reached when cupric sulfate is added at a concentration at least about two-times of thioredoxin concentration in the pre-harves or harvested culture fluid.

In different embodiment, the thioredoxin inhibitor is an oxidizing agent, e.g., an inhibitor of G6PD, such as, for example, pyridoxal 5'-phosphate, 1 fluoro-2,4 dinitrobenzene, dehydroepiandrosterone (DHEA) or epiandrosterone (EA); cystine or cysteine. Typical effective inhibitor concen-

trations of DHEA are between about 0.05 mM and about 5 mM, or between about 0.1 mM and about 2.5 mM.

In a further embodiment, the thioredoxin inhibitor is an inhibitor of hexokinase activity, including, without limitation, chelators of metal ions, such as, for example, ethylenediamine tetraacetic acid (EDTA). EDTA is typically added and effective at a concentration between about 5 mM and about 60 mM, or about 10 mM and about 50 mM, or about 20 mM and about 40 mM.

In other preferred embodiments, the inhibitor of hexokinase activity is selected from the group consisting of sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose, 2-C-hydroxy-methylglucose, xylose, and lyxose.

Other inhibitors include cystine, cysteine, and oxidized glutathione which are typically added at a concentration at least about 40-times of the concentration of the polypeptide in question in the pre-harvest or harvested culture fluid.

In a still further embodiment, the thioredoxin inhibitor is an siRNA, an antisense nucleotide, or an antibody specifically binding to a thioredoxin reductase.

In another embodiment, the thioredoxin inhibitor is a measure indirectly resulting in the inhibition of thioredoxin activity. This embodiment includes, for example, air sparging the harvested culture fluid of the recombinant host cell, and/or lowering the pH of the harvested culture fluid of the recombinant host cell.

In various embodiments, indirect means for inhibiting thioredoxin activity, such as air sparging and/or lowering of the pH, can be combined with the use of direct thioredoxin inhibitors, such as those listed above.

In all embodiments, the polypeptide may, for example, be an antibody, or a biologically functional fragment of an antibody. Representative antibody fragments include Fab, Fab', $F(ab')_2$, scFv, $(scFv)_2$, dAb, complementarity determining region (CDR) fragments, linear antibodies, single-chain antibody molecules, minibodies, diabodies, and multispecific antibodies formed from antibody fragments.

Therapeutic antibodies include, without limitation, anti-HER2 antibodies anti-CD20 antibodies; anti-IL-8 antibodies; anti-VEGF antibodies; anti-CD40 antibodies, anti-CD11a antibodies; anti-CD18 antibodies; anti-IgE antibodies; anti-Apo-2 receptor antibodies; anti-Tissue Factor (TF) antibodies; anti-human $\alpha_4\beta_7$ integrin antibodies; anti-EGFR antibodies; anti-CD3 antibodies; anti-CD25 antibodies; anti-CD4 antibodies; anti-CD52 antibodies; anti-Fc receptor antibodies; anti-carcinoembryonic antigen (CEA) antibodies; antibodies directed against breast epithelial cells; antibodies that bind to colon carcinoma cells; anti-CD38 antibodies; anti-CD33 antibodies; anti-CD22 antibodies; anti-EpCAM antibodies; anti-GpIIb/IIIa antibodies; anti-RSV antibodies; anti-CMV antibodies; anti-HIV antibodies; anti-hepatitis antibodies; anti-CA 125 antibodies; anti-$\alpha v\beta 3$ antibodies; anti-human renal cell carcinoma antibodies; anti-human 17-1A antibodies; anti-human colorectal tumor antibodies; anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma; and anti-human leukocyte antigen (HLA) antibodies, and anti-HLA DR antibodies.

In other embodiments, the therapeutic antibody is an antibody binding to a HER receptor, VEGF, IgE, CD20, CD11a, CD40, or DR5.

In a further embodiment, the HER receptor is HER1 and/or HER2, preferably HER2. The HER2 antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NO: 16, 17, 18, and 19.

In another embodiment, the therapeutic antibody is an antibody that binds to CD20. The anti-CD20 antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NOS: 1 through 15.

In yet another embodiment, the therapeutic antibody is an antibody that binds to VEGF. The anti-VEGF antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NOS: 20 through 25.

In an additional embodiment, the therapeutic antibody is an antibody that binds CD11a. The anti-CD11a antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NOS: 26 through 29.

In a further embodiment, the therapeutic antibody binds to a DR5 receptor. The anti-DR5 antibody may, for example, be selected from the group consisting of Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3, and preferably is Apomab 8.3 or Apomab 7.3, and most preferably Apomab 7.3.

In other embodiments of the method of the present invention, the polypeptide expressed in the recombinant host cell is a therapeutic polypeptide. For example, the therapeutic polypeptide can be selected from the group consisting of a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an

ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of said polypeptides.

In all embodiments, the recombinant host cell can be an eukaryotic host cell. such as a mammalian host cell, including, for example, Chinese Hamster Ovary (CHO) cells.

In all embodiments, the recombinant host cell can also be a prokaryotic host cell, such as a bacterial cell, including, without limitation, *E. coli* cells.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Dialysis Experiment: Digital gel-like imaging obtained from Bioanalyzer analysis (each lane representing a time point) demonstrating that ocrelizumab (rhuMAb 2H7—Variant A) inside the dialysis bag remained intact during the incubation period.

FIG. **2**. Dialysis Experiment: Digital gel-like imaging obtained from Bioanalyzer analysis (each lane representing a time point) showing that ocrelizumab outside the dialysis bag was reduced during the incubation period. This is evidenced by the loss of intact antibody (~150 kDa) and the formation of antibody fragments depicted in the Figure. At the 48-hour time point (Lane 7), the reduced antibody appeared to be reoxidized, presumably as a result of loosing reduction activity in the Harvested Cell Culture Fluid (HCCF). The band appearing just above the 28 kDa marker arose from the light chain of antibody. There was a significant amount of free light already present in the HCCF before the incubation began. The presence of excess free light chain and dimers of light chain in the HCCF is typical for the cell line producing ocrelizumab.

FIG. **3**. Free Thiol Levels from Dialysis Experiment: Purified ocrelizumab in phosphate buffered saline (PBS) was inside the dialysis bag and HCCF containing ocrelizumab was outside the bag. Free thiols inside (boxes) and outside (diamonds) the dialysis bag reached comparable levels within a few hours, indicating a good exchange of small molecule components in the HCCF between inside and outside the dialysis bag.

FIG. **4**. Thioredoxin System and Other Reactions Involved in Antibody Reduction: The thioredoxin system, comprising thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH, functions as a hydrogen donor system for reduction of disulfide bonds in proteins. Trx is a small monomeric protein with a COX active site motif that catalyzes many redox reactions through thiol-disulfide exchange. The oxidized Trx can be reduced by NADPH via TrxR. The reduced Trx is then able to catalyze the reduction of disulfides in proteins. The NADPH required for thioredoxin system is provided via reactions in pentose phosphate pathway and glycolysis.

FIG. **5**. In Vitro Activity of Thioredoxin System: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) demonstrating that incubation of intact ocrelizumab (1 mg/mL) with 0.1 mM TrxR (rat liver), 5 mM Trx (human), and 1 mM NADPH in PBS resulted in the complete reduction of ocrelizumab; the ocrelizumab was completely reduced in less than 21 hours.

FIG. **6**. In Vitro Activity of Thioredoxin System Inhibited by Aurothioglucose: The addition of aurothioglucose (ATG) to the same reaction mixture as described in the caption for FIG. **5**, above, effectively inhibited the ocrelizumab reduction. This is seen by the digital gel-like image from Bioanalyzer analysis (each lane representing a time point).

FIG. **7**. In vitro Activity of Thioredoxin System Inhibited by Aurothiomalate: The addition of aurothiomalate (ATM) at a concentration of 1 mM to the same reaction mixture as described in the caption for FIG. **5**, above, effectively inhibited the ocrelizumab reduction. This is seen by the digital gel-like image from Bioanalyzer analysis (each lane representing a time point).

FIG. **8**. In Vitro Activity of Thioredoxin System: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that incubation of intact ocrelizumab (1 mg/mL) with 0.1 mM TrxR (rat liver), 5 mM Trx (human), and 1 mM NADPH in 10 mM histidine sulfate buffer resulted in the reduction of ocrelizumab in less than 1 hour.

FIG. **9**. In vitro Activity of Thioredoxin System Inhibited by $CuSO_4$: The addition of $CuSO_4$ at a concentration of 50 μM to the same reaction mixture as described in the caption for FIG. **8** effectively inhibited the ocrelizumab reduction as shown in the digital gel-like image from Bioanalyzer analysis (each lane representing a time point).

FIG. **10**. Ocrelizumab Reduction: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that ocrelizumab was reduced in an incubation experiment using HCCF from a homogenized CCF generated from a 3-L fermentor.

FIG. **11**. Inhibition of Ocrelizumab Reduction In HCCF by Aurothioglucose: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that the addition of 1 mM aurothioglucose to the same HCCF as used for the incubation experiment as shown in FIG. **10** inhibited the reduction of ocrelizumab.

FIG. **12**. Inhibition of Ocrelizumab Reduction In HCCF by Aurothiomalate: Digital gel-like image from Bioanalyzer (each lane representing a time point) analysis indicating that the addition of 1 mM aurothiomalate to the same HCCF as used for the incubation experiment shown in FIG. **10** inhibited the reduction of ocrelizumab.

FIG. **13**. Losing Reduction Activity in HCCF: The HCCF from one of the large scale manufacturing runs for ocrelizumab (the "beta" run) that was subject to several freeze/thaw cycles demonstrated no ocrelizumab reduction when used in an incubation experiment. This was shown by Bioanalyzer analysis (each lane representing a time point), and can be contrasted to the antibody reduction seen previously in the freshly thawed HCCF from the same fermentation batch.

FIG. **14**. The Lost Reduction Activity in HCCF Restored by Addition of NADPH: The reduction of ocrelizumab was observed again in the Bioanalyzer assay (each lane representing a time point) after the addition of NADPH at a concentration of 5 mM into the HCCF where the reduction activity has been eliminated under the conditions described above in FIG. **13**.

FIG. **15**. The Lost Reduction Activity in HCCF Restored by Addition of Glucose-6-Phosphate: The reduction of ocrelizumab was observed again in the Bioanalyzer assay (each lane representing a time point) after the addition of G6P at a concentration of 10 mM into the HCCF where the reduction activity has been eliminated due to the treatment described above in FIG. **13**.

FIG. **16**. Ocrelizumab Reduction: A digital gel-like image from Bioanalyzer analysis showing that ocrelizumab was reduced in an incubation experiment using a HCCF from a large scale manufacturing run (the "alpha" run).

FIG. **17**. EDTA Inhibits Ocrelizumab Reduction: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that the reduction of ocrelizumab was inhibited in an incubation experiment using a HCCF from the alpha run with EDTA added at a concentration of 20 mM to the HCCF whose reducing activity is demonstrated in FIG. **16**.

FIG. **18**. The Lost Reduction Activity in "Beta Run" HCCF Restored by Addition of Glucose-6-Phosphate but No Inhibition of Reduction by EDTA: The reduction of ocrelizumab was observed in the Bioanalyzer assay (each lane representing a time point) after the addition of G6P at a concentration of 5 mM and 20 mM EDTA into the HCCF whose reduction activity had been lost (see FIG. **13**). In contrast to the results shown in FIG. **17**, the presence of EDTA did not block the reduction of ocreliumab.

FIG. **19**. Inhibition of Ocrelizumab Reduction: by (i) addition of EDTA, (ii) addition of $CuSO_4$, or (iii) adjustment of pH to 5.5. All three different methods, (1) addition of EDTA, (2) addition of $CuSO_4$, and (3) adjustment of pH to 5.5, used independently, were effective in inhibiting ocrelizumab reduction. This was demonstrated by the depicted quantitative Bioanalyzer results that showed that nearly 100% intact (150 kDa) antibody remained in the protein A elution pools. In contrast, ocrelizumab was completely reduced in the control HCCF after 20 hours of HCCF hold time.

FIG. **20**. Inhibition of Ocrelizumab Reduction by Air Sparging: Sparging the HCCF with air was effective in inhibiting ocrelizumab disulfide bond reduction. This was demonstrated by the quantitative Bioanalyzer results showing that nearly 100% intact (150 kDa) antibody remained in the protein A elution pools. In contrast, ocrelizumab was almost completely reduced in the control HCCF after 5 hours of sparging with nitrogen.

FIG. **21** shows the V_L (SEQ ID NO. 24) amino acid sequence of an anti-Her2 antibody (Trastuzumab).

FIG. **22** shows the V_H (SEQ ID No. 25) amino acid sequence of an anti-Her2 antibody (Trastuzumab).

FIG. **23** is a schematic showing some steps of a typical large scale manufacturing process.

FIG. **24** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate.

FIG. **25** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 1 mM histidine sulfate+1 mM ATG.

FIG. **26** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.6 μM ATG (6:1 ATG:TrxR).

FIG. **27** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.4 μM ATG (4:1 ATG:TrxR).

FIG. **28** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.2 μM ATG (2:1 ATG:TrxR).

FIG. **29** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.1 mM autothiomalate (ATM).

FIG. **30** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.01 mM autothiomalate (ATM).

FIG. **31** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+20 μM $CuSO_4$ (4:1 Cu^{2+}:Trx).

FIG. **32** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+10 μM $CuSO_4$ (2:1 Cu^{2+}:Trx).

FIG. **33** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+5 μM $CuSO_4$ (1:1 Cu^{2+}:Trx).

FIG. **34** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+532 μM cystamine (20:1 cystamine:2H7 disulfide).

FIG. **35** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+266 μM cystamine (10:1 cystamine:2H7 disulfide).

FIG. **36** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+133 μM cystamine (5:1 cystamine:2H7 disulfide).

FIG. **37** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+26.6 μM cystamine (1:1 cystamine:2H7 disulfide).

FIG. **38** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate (pH=7.6)+2.6 mM cystine.

FIG. **39** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+2.6 mM GSSG (oxidized glutathione).

FIG. **40** Reconstructed enzymatic reduction system. 1 mg/ml 2H7 (Variant A)+10 μg/mL hexokinase, 50 μg/mL glucose-6-phosphate dehydrogenase, 5 μM thioredoxin, 0.1 μM thioredoxin reductase, 2 mM glucose, 0.6 mM ATP, 2 mM Mg^{2+}, and 2 mM NADP in 50 mM histidine sulfate buffer at pH=7.38.

FIG. **41** The thioredoxin system requires NADPH. 1 mg/ml 2H7 (Variant A)+5 μM thioredoxin, 0.1 μM thioredoxin reductase, and 2 mM NADP in 50 mM histidine sulfate buffer at pH=7.38.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

In the present invention, in the context of proteins, including antibodies, in general, or with regard to any specific protein or antibody, the term "reduction" is used to refer to the reduction of one or more disulfide bonds of the protein or antibody. Thus, for example, the terms "ocrelizumab reduction" is used interchangeably with the term "ocrelizumab disulfide bond reduction" and the term "antibody (Ab) reduction" is used interchangeably with the term "antibody (Ab) disulfide bond reuction."

The terms "reduction" or "disulfide bond reduction" are used in the broadest sense, and include complete and partial reduction and reduction of some or all of the disulfide bonds, interchain or intrachain, present in a protein such as an antibody.

By "protein" is meant a sequence of amino acids for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. This is to distinguish from "peptides" or other small molecular weight drugs that do not have such structure. Typically, the protein herein will have a molecular weight of at least about 15-20 kD, preferably at least about 20 kD. Examples of proteins encompassed

within the definition herein include all mammalian proteins, in particular, therapeutic and diagnostic proteins, such as therapeutic and diagnostic antibodies, and, in general proteins that contain one or more disulfide bonds, including multi-chain polypeptides comprising one or more inter- and/or intrachain disulfide bonds.

The term "therapeutic protein" or "therapeutic polypeptide" refers to a protein that is used in the treatment of disease, regardless of its indication or mechanism of action. In order for therapeutic proteins to be useful in the clinic it must be manufactured in large quantities. "Manufacturing scale" production of therapeutic proteins, or other proteins, utilize cell cultures ranging from about 400 L to about 80,000 L, depending on the protein being produced and the need. Typically such manufacturing scale production utilizes cell culture sizes from about 400 L to about 25,000 L. Within this range, specific cell culture sizes such as 4,000 L, about 6,000 L, about 8,000, about 10,000, about 12,000 L, about 14,000 L, or about 16,000 L are utilized.

The term "therapeutic antibody" refers to an antibody that is used in the treatment of disease. A therapeutic antibody may have various mechanisms of action. A therapeutic antibody may bind and neutralize the normal function of a target associated with an antigen. For example, a monoclonal antibody that blocks the activity of the of protein needed for the survival of a cancer cell causes the cell's death. Another therapeutic monoclonal antibody may bind and activate the normal function of a target associated with an antigen. For example, a monoclonal antibody can bind to a protein on a cell and trigger an apoptosis signal. Yet another monoclonal antibody may bind to a target antigen expressed only on diseased tissue; conjugation of a toxic payload (effective agent), such as a chemotherapeutic or radioactive agent, to the monoclonal antibody can create an agent for specific delivery of the toxic payload to the diseased tissue, reducing harm to healthy tissue. A "biologically functional fragment" of a therapeutic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

The term "diagnostic protein" refers to a protein that is used in the diagnosis of a disease.

The term "diagnostic antibody" refers to an antibody that is used as a diagnostic reagent for a disease. The diagnostic antibody may bind to a target antigen that is specifically associated with, or shows increased expression in, a particular disease. The diagnostic antibody may be used, for example, to detect a target in a biological sample from a patient, or in diagnostic imaging of disease sites, such as tumors, in a patient. A "biologically functional fragment" of a diagnostic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

"Purified" means that a molecule is present in a sample at a concentration of at least 80-90% by weight of the sample in which it is contained.

The protein, including antibodies, which is purified is preferably essentially pure and desirably essentially homogeneous (i.e. free from contaminating proteins etc.).

An "essentially pure" protein means a protein composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight.

An "essentially homogeneous" protein means a protein composition comprising at least about 99% by weight of protein, based on total weight of the composition.

As noted above, in certain embodiments, the protein is an antibody. "Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which generally lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region), antibody compositions with polyepitopic specificity, bispecific antibodies, diabodies, and single-chain molecules such as scFv molecules, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible mutations, e.g., naturally occurring mutations, that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. In certain embodiments, such a monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins.

The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler et al., *Nature,* 256: 495 (1975); Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567), phage display technologies (see, e.g., Clackson et al., *Nature,* 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1992); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101 (34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284(1-2): 119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes

encoding human immunoglobulin sequences (see, e.g., WO98/24893; WO96/34096; WO96/33735; WO91/10741; Jakobovits et al., *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits et al., *Nature* 362: 255-258 (1993); Bruggemann et al., *Year in Immunol.* 7:33 (1993); U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016; Marks et al., *BioTechnology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild et al., *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996) and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. In one embodiment, a humanized antibody is a human immunoglobulin (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and/or capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications may be made to further refine antibody performance. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin, and all or substantially all the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also the following review articles and references cited therein: Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994). The humanized antibody includes a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs/HVRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al., Bio/Technology 10:779-783 (1992) describes affinity maturation by V_H and V_L domain shuffling. Random mutagenesis of CDR/HVR and/or framework residues is described by: Barbas et al., *Proc Nat. Acad. Sci. USA* 91:3809-3813 (1994); Schier et al., *Gene* 169:147-155 (1995); Yelton et al., *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al., *J. Mol. Biol.* 226:889-896 (1992).

The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "V_H." The variable domain of the light chain may be referred to as "V_L." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions (HVRs) both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a beta-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest*, Fifth Edition, National Institute of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in the binding of an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called a, d, e, g, and m, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas et al., Cellular and *Mol. Immunology,* 4th ed. (2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain the Fc region.

"Antibody fragments" comprise only a portion of an intact antibody, wherein the portion retains at least one, and as many

as most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such an antibody fragment may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')2 antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

"Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404,097; WO93/1161; Hudson et al., (2003) *Nat. Med.* 9:129-134; and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., (2003) *Nat. Med.* 9:129-134.

The antibody may bind to any protein, including, without limitation, a member of the HER receptor family, such as HER1 (EGFR), HER2, HER3 and HER4; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD21, CD22, and CD34; cell adhesion molecules such as LFA-1, Mol, p150, 95, VLA-4, ICAM-1, VCAM and av/p3 integrin including either α or β or subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as vascular endothelial growth factor (VEGF); IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; and protein C. Other exemplary proteins include growth hormone (GH), including human growth hormone (hGH) and bovine growth hormone (bGH); growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA); bombazine; thrombin; tumor necrosis factor-α and -β; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); serum albumin such as human serum albumin (HSA); mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins (IGFBPs); erythropoietin (EPO); thrombopoietin (TPO); osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and biologically active fragments or variants of any of the above-listed polypeptides. Many other antibodies and/or other proteins may be used in accordance with the instant invention, and the above lists are not meant to be limiting.

A "biologically functional fragment" of an antibody comprises only a portion of an intact antibody, wherein the portion retains at least one, and as many as most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, a biologically functional fragment of an antibody comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, a biologically functional fragment of an antibody, for example one that comprises the Fc region, retains at least one of the biological functions normally asso-

ciated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, a biologically functional fragment of an antibody is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such a biologically functional fragment of an antibody may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

The terms “thioredoxin inhibitor” and “Trx inhibitor” are used interchangeably, and include all agents and measures effective in inhibiting thioredoxin activity. Thus, thioredoxin (Trx) inhibitors include all agents and measures blocking any component of the Trx, G6PD and/or hexokinase enzyme systems. In this context, “inhibition” includes complete elimination (blocking) and reduction of thioredoxin activity, and, consequently, complete or partial elimination of disulfide bond reduction in a protein, such as an antibody.

An “isolated” antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater than 99% by weight; (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody’s natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The terms “Protein A” and “ProA” are used interchangeably herein and encompasses Protein A recovered from a native source thereof, Protein A produced synthetically (e.g. by peptide synthesis or by recombinant techniques), and variants thereof which retain the ability to bind proteins which have a C_H2/C_H3 region, such as an Fc region. Protein A can be purchased commercially from Repligen, GE Healthcare and Fermatech. Protein A is generally immobilized on a solid phase support material. The term “ProA” also refers to an affinity chromatography resin or column containing chromatographic solid support matrix to which is covalently attached Protein A.

The term “chromatography” refers to the process by which a solute of interest in a mixture is separated from other solutes in a mixture as a result of differences in rates at which the individual solutes of the mixture migrate through a stationary medium under the influence of a moving phase, or in bind and elute processes.

The term “affinity chromatography” and “protein affinity chromatography” are used interchangeably herein and refer to a protein separation technique in which a protein of interest or antibody of interest is reversibly and specifically bound to a biospecific ligand. Preferably, the biospecific ligand is covalently attached to a chromatographic solid phase material and is accessible to the protein of interest in solution as the solution contacts the chromatographic solid phase material. The protein of interest (e.g., antibody, enzyme, or receptor protein) retains its specific binding affinity for the biospecific ligand (antigen, substrate, cofactor, or hormone, for example) during the chromatographic steps, while other solutes and/or proteins in the mixture do not bind appreciably or specifically to the ligand. Binding of the protein of interest to the immobilized ligand allows contaminating proteins or protein impurities to be passed through the chromatographic medium while the protein of interest remains specifically bound to the immobilized ligand on the solid phase material. The specifically bound protein of interest is then removed in active form from the immobilized ligand with low pH, high pH, high salt, competing ligand, and the like, and passed through the chromatographic column with the elution buffer, free of the contaminating proteins or protein impurities that were earlier allowed to pass through the column. Any component can be used as a ligand for purifying its respective specific binding protein, e.g. antibody.

The terms “non-affinity chromatography” and “non-affinity purification” refer to a purification process in which affinity chromatography is not utilized. Non-affinity chromatography includes chromatographic techniques that rely on non-specific interactions between a molecule of interest (such as a protein, e.g. antibody) and a solid phase matrix.

A “cation exchange resin” refers to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin may, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-methyl-cellulose, sulphopropyl (SP) immobilized on agarose (e.g. SP-SEPHAROSE FAST FLOW™ or SP-SEPHAROSE HIGH PERFORMANCE™, from GE Healthcare) and sulphonyl immobilized on agarose (e.g. S-SEPHAROSE FAST FLOW™ from GE Healthcare). A “mixed mode ion exchange resin” refers to a solid phase which is covalently modified with cationic, anionic, and hydrophobic moieties. A commercially available mixed mode ion exchange resin is BAKERBOND ABX™ (J. T. Baker, Phillipsburg, N.J.) containing weak cation exchange groups, a low concentration of anion exchange groups, and hydrophobic ligands attached to a silica gel solid phase support matrix.

The term “anion exchange resin” is used herein to refer to a solid phase which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Commercially available anion exchange resins include DEAE cellulose, QAE SEPHADEX™ and FAST Q SEPHAROSE™ (GE Healthcare).

A “buffer” is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in *Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., ed. Calbiochem Corporation (1975). In one embodiment, the buffer has a pH in the range from about 2 to about 9, alternatively from about 3 to about 8, alternatively from about 4 to about 7 alternatively from about 5 to about 7. Non-limiting examples of buffers that will control the pH in this range include MES, MOPS, MOPSO, Tris, HEPES, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

The “loading buffer” is that which is used to load the composition comprising the polypeptide molecule of interest and one or more impurities onto the ion exchange resin. The loading buffer has a conductivity and/or pH such that the polypeptide molecule of interest (and generally one or more impurities) is/are bound to the ion exchange resin or such that the protein of interest flows through the column while the impurities bind to the resin.

The "intermediate buffer" is used to elute one or more impurities from the ion exchange resin, prior to eluting the polypeptide molecule of interest. The conductivity and/or pH of the intermediate buffer is/are such that one or more impurity is eluted from the ion exchange resin, but not significant amounts of the polypeptide of interest.

The term "wash buffer" when used herein refers to a buffer used to wash or re-equilibrate the ion exchange resin, prior to eluting the polypeptide molecule of interest. Conveniently, the wash buffer and loading buffer may be the same, but this is not required.

The "elution buffer" is used to elute the polypeptide of interest from the solid phase. The conductivity and/or pH of the elution buffer is/are such that the polypeptide of interest is eluted from the ion exchange resin.

A "regeneration buffer" may be used to regenerate the ion exchange resin such that it can be re-used. The regeneration buffer has a conductivity and/or pH as required to remove substantially all impurities and the polypeptide of interest from the ion exchange resin.

The term "substantially similar" or "substantially the same," as used herein, denotes a sufficiently high degree of similarity between two numeric values (for example, one associated with an antibody of the invention and the other associated with a reference/comparator antibody), such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, and/or less than about 10% as a function of the reference/comparator value.

The phrase "substantially reduced," or "substantially different," as used herein with regard to amounts or numerical values (and not as reference to the chemical process of reduction), denotes a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, and/or greater than about 50% as a function of the value for the reference/comparator molecule.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors," or simply, "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector.

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, Calif., or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and

where Y is the total number of amino acid residues in B.

It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

"Percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a reference Factor D-encoding sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. Sequence identity is then calculated relative to the longer sequence, i.e. even if a shorter

sequence shows 100% sequence identity with a portion of a longer sequence, the overall sequence identity will be less than 100%.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. "Treatment" herein encompasses alleviation of the disease and of the signs and symptoms of the particular disease.

A "disorder" is any condition that would benefit from treatment with the protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include carcinomas and allergies.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, non-human higher primates, other vertebrates, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

An "interfering RNA" or "small interfering RNA (siRNA)" is a double stranded RNA molecule less than about 30 nucleotides in length that reduces expression of a target gene. Interfering RNAs may be identified and synthesized using known methods (Shi Y., Trends in Genetics 19(1):9-12 (2003), WO/2003056012 and WO2003064621), and siRNA libraries are commercially available, for example from Dharmacon, Lafayette, Colo. Frequently, siRNAs can be successfully designed to target the 5' end of a gene.

II. Compositions and Methods of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology and the like, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., *Molecular Cloning: A Laboratory Manual*, (J. Sambrook et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989); *Current Protocols in Molecular Biology* (F. Ausubel et al., eds., 1987 updated); *Essential Molecular Biology* (T. Brown ed., IRL Press 1991); *Gene Expression Technology* (Goeddel ed., Academic Press 1991); *Methods for Cloning and Analysis of Eukaryotic Genes* (A. Bothwell et al., eds., Bartlett Publ. 1990); *Gene Transfer and Expression* (M. Kriegler, Stockton Press 1990); *Recombinant DNA Methodology II* (R. Wu et al., eds., Academic Press 1995); PCR: *A Practical Approach* (M. McPherson et al., IRL Press at Oxford University Press 1991); *Oligonucleotide Synthesis* (M. Gait ed., 1984); *Cell Culture for Biochemists* (R. Adams ed., Elsevier Science Publishers 1990); *Gene Transfer Vectors for Mammalian Cells* (J. Miller & M. Calos eds., 1987); *Mammalian Cell Biotechnology* (M. Butler ed., 1991); *Animal Cell Culture* (J. Pollard et al., eds., Humana Press 1990); *Culture of Animal Cells*, 2nd Ed. (R. Freshney et al., eds., Alan R. Liss 1987); *Flow Cytometry and Sorting* (M. Melamed et al., eds., Wiley-Liss 1990); the series *Methods in Enzymology* (Academic Press, Inc.); Wirth M. and Hauser H. (1993); *Immunochemistry in Practice*, 3rd edition, A. Johnstone & R. Thorpe, Blackwell Science, Cambridge, Mass., 1996; *Techniques in Immunocytochemistry*, (G. Bullock & P. Petrusz eds., Academic Press 1982, 1983, 1985, 1989); Handbook of Experimental Immunology, (D. Weir & C. Blackwell, eds.); *Current Protocols in Immunology* (J. Coligan et al., eds. 1991); *Immunoassay* (E. P. Diamandis & T. K. Christopoulos, eds., Academic Press, Inc., 1996); Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed) Academic Press, New York; Ed Harlow and David Lane, *Antibodies A laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988; *Antibody Engineering*, 2nd edition (C. Borrebaeck, ed., Oxford University Press, 1995); and the series *Annual Review of Immunology*; the series *Advances in Immunology*.

1. Prevention of Disulfide Bond Reduction

The present invention concerns methods for the prevention of the reduction of disulfide bonds of proteins during recombinant production. In particular, the invention concerns methods for preventing the reduction of disulfide bonds of recombinant proteins during processing following fermentation. The methods of the invention are particularly valuable for large scale production of disulfide bond containing proteins, such as at a manufacturing scale. In one embodiment, the methods of the invention are useful for large scale protein production at a scale of greater than 5,000 L.

It has been experimentally found that disulfide bond reduction occurs during processing of the Harvested Cell Culture Fluid (HCCF) produced during manufacturing of recombinant proteins that contain disulfide bonds. Typically, this reduction is observed after cell lysis, especially mechanical cell lysis during harvest operations, when it reaches a certain threshold, such as, for example, from about 30% to about 70%, or from about 40% to about 60%, or from about 50% to about 60% total cell lysis. This threshold will vary, depending on the nature of the protein (e.g. antibody) produced, the recombinant host, the production system, production parameters used, and the like, and can be readily determined experimentally.

Theoretically, such reduction might result from a variety of factors and conditions during the manufacturing process, and might be caused by a variety of reducing agents. The present invention is based, at least in part, on the recognition that the root cause of this reduction is an active thioredoxin (Trx) or thioredoxin-like system in the HCCF.

The Trx enzyme system, composed of Trx, thioredoxin reductase (TrxR) and NADPH, is a hydrogen donor system for reduction of disulfide bonds in proteins. Trx is a small monomeric protein with a COX active site motif that catalyzes many redox reactions through thiol-disulfide exchange. The oxidized Trx can be reduced by NADPH via TrxR. The reduced Trx is then able to catalyze the reduction of disulfides in proteins. The NADPH required for thioredoxin system is provided via reactions in pentose phosphate pathway and glycolysis. The results presented herein demonstrate that NADPH, which is required for activity of the Trx system is provided by glucose-6-phosphate dehyrogenase (G6PD) activity, which generates NADPH from glucose and ATP by hexokinase (see FIG. **4**). These cellular enzymes (Trx system, G6PD, and hexokinase) along with their substrates are released into the CCF upon cell lysis, allowing reduction to occur. Accordingly, disulfide reduction can be prevented by inhibitors of the Trx enzyme system or upstream enzyme systems providing components for an active Trx system, such as G6PD and hexokinase activity.

For further details of these enzyme systems, or regarding other details of protein production, see, for example: Babson, A. L. and Babson, S. R. (1973) Kinetic Colorimetric Measurement of Serum Lactate Dehydrogenase Activity. *Clin. Chem.* 19: 766-769; Michael W. Laird et al., "Optimization of BLyS Production and Purification from *Eschericia coli*," *Protein Expression and Purification* 39:237-246 (2005); John C. Joly et al., "Overexpression of *Eschericia coli* Oxidoreductases Increases Recombinant Insulin-like Growth Factor-I Accumulation," Proc. Natl. Acad. Sci. USA 95:2773-2777 (March 1998); Dana C. Andersen et al., "Production Technologies for Monoclonal Antibodies and Their Fragments,"

Current Opinion in Biotechnology 15:456-462 (2004); Yariv Mazor et al., "Isolation of Engineered, Full-length Antibodies from Libraries Expressed in *Escherichia coli," Nature Biotech.* 25, 563-565 (1 Jun. 2007); Laura C. Simmons et al., "Expression of Full-length Immunoglobulins in *Escherichia coli*: Rapid and Efficient Production of Aglycosylated Antibodies," *Journal of Immunological Methods* 263:133-147 (2002); Paul H. Bessette et al., "Efficient Folding of Proteins with Multiple Disulfide Bonds in the *Escherichia coli* cytoplasm," Proc. Natl. Acad. Sci. 96(24):13703-08 (1999); Chaderjian, W. B., Chin, E. T., Harris, R. J., and Etcheverry, T. M., (2005) "Effect of copper sulfate on performance of a serum-free CHO cell culture process and the level of free thiol in the recombinant antibody expressed," *Biotechnol. Prog.* 21: 550-553; Gordon G., Mackow M. C., and Levy H. R., (1995) "On the mechanism of interaction of steroids with human glucose 6-phosphate dehydrogenase," Arch. Biochem. Biophys. 318: 25-29; Gromer S., Urig S., and Becker K., (2004) "TheTrx System—From Science to Clinic," Medicinal Research Reviews, 24: 40-89; Hammes G. G. and Kochavi D., (1962a) "Studies of the Enzyme Hexokinase. I. Steady State Kinetics at pH 8," J. Am. Chem. Soc. 84:2069-2073; Hammes G. G. and Kochavi D., (1962b) "Studies of the Enzyme Hexokinase. III. The Role of the Metal Ion," J. Am. Chem. Soc. 84:2076-2079; Johansson C., Lillig C. H., and Holmgren A., (2004) "Human Mitochondrial Glutaredoxin Reduces S-Glutathionylated Proteins with High Affinity Accepting Electrons from Either Glutathione or Thioredoxin Reductase," J. Biol. Chem. 279:7537-7543; Legrand, C., Bour, J. M., Jacob, C., Capiaumont J., Martial, A., Marc, A., Wudtke, M., Kretzmer, G., Demangel, C., Duval, D., and Hache J., (1992) "Lactate Dehydrogenase (LDH) Activity of the Number of Dead Cells in the Medium of Cultured Eukaryotic Cells as Marker," J. Biotechnol., 25: 231-243; McDonald, M. R., (1955) "Yeast Hexokinase: ATP+ Hexose→Hexose-6-phosphate+ADP," Methods in Enzymology, 1: 269-276, Academic Press, NY; Sols, A., DelaFuente, G., Villar-Palasi, C., and Asensio, C., (1958) "Substrate Specificity and Some Other Properties of Bakers' Yeast Hexokinase," Biochim Biophys Acta 30: 92-101; Kirkpatrick D. L., Kuperus M., Dowdeswell M., Potier N., Donald L. J., Kunkel M., Berggren M., Angulo M., and Powis G., (1998) "Mechanisms of inhibition of the Trx growth factor system by antitumor 2-imidazolyl disulfides," Biochem. Pharmacol. 55: 987-994; Kirkpatrick D. L., Watson S., Kunkel M., Fletcher S., Ulhaq S., and Powis G., (1999) "Parallel syntheses of disulfide inhibitors of the Trx redox system as potential antitumor agents," Anticancer Drug Des. 14: 421-432; Milhausen, M., and Levy, H. R., (1975) "Evidence for an Essential Lysine in G6PD from *Leuconostoc mesenteroides*," Eur. J. Biochem. 50: 453-461; Pleasants, J. C., Guo, W., and Rabenstein, D. L., (1989) "A comparative study of the kinetics of selenol/diselenide and thiol/disulfide exchange reactions," J. Am. Chem. Soc. 111: 6553-6558; Whitesides, G. M., Lilburn, J. E., and Szajewski, R. P., (1977) "Rates of thioldisulfide interchange reactions between mono- and dithiols and Ellman's reagent," J. Org. Chem. 42: 332-338; and Wipf P., Hopkins T. D., Jung J. K., Rodriguez S., Birmingham A., Southwick E. C., Lazo J. S., and Powis G, (2001) "New inhibitors of the Trx-TrxR system based on a naphthoquinone spiroketal natural product lead," *Bioorg. Med. Chem. Lett.* 11: 2637-2641.

According to one aspect of the present invention, disulfide bond reduction can be prevented by blocking any component of the Trx, G6PD and hexokinase enzyme systems. Inhibitors of these enzyme systems are collectively referred to herein as "thioredoxin inhibitors," or "Trx inhibitors." The Trx inhibitors are typically added to the cell culture fluid (CCF), which contains the recombinant host cells and the culture media, and/or to the harvested cell culture fluid (HCCF), which is obtained after harvesting by centrifugation, filtration, or similar separation methods. The HCCF lacks intact host cells but typically contains host cell proteins and other contaminants, including DNA, which are removed in subsequent purification steps. Thus, the Trx inhibitors may be added before harvest and/or during harvest, preferably before harvest.

Alternatively or in addition other, non-specific methods can also be used to prevent the reduction of disulfide bond reduction following fermentation during the recombinant production of recombinant proteins, such as air sparging or pH adjustment. Certain reduction inhibition methods contemplated herein are listed in the following Table 1.

TABLE 1

Reduction Inhibition Methods

Method[1]	Purpose
Addition of EDTA, EGTA, or citrate	To inhibit hexokinase
Addition of sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose, 2-C-hydroxy-methylglucose, xylose, or lyxose	To inhibit hexokinase
Addition of epiandrosterone or dehydroepiandrosterone (DHEA)	To inhibit G6PD
Addition of pyridoxal 5'-phosphate or 1-fluoro-2,4-dinitrobenzene	To inhibit G6PD
Addition of metal ions such as Cu^{2+}, Zn^{2+} Hg^{2+}, Co^{2+}, or Mn^{2+}	To inhibit Trx system
Addition of alkyl-2-imidazolyl disulfides and related compounds (e.g., 1 methylpropyl-2-imidazolyl disulfide[2]) or naphthoquinone spiroketal derivatives (e.g. palmarumycin CP_1[2])	To inhibit Trx
Addition of aurothioglucose (ATG) or aurothiomalate (ATM)	To inhibit TrxR
Air sparging	To deplete G6P and NADPH; oxidizing agent
Cystine	Oxidizing agent
Oxidized glutathione	Oxidizing agents
pH Adjustment to below 6.0	To reduce thiol-disulfide exchange rate and Trx system activity

[1]Applied to CCF prior to harvest or in HCCF immediately after harvest.
[2]Currently not available commercially.

"Trx inhibitors" for use in the methods of the present invention include, without limitation, (1) direct inhibitors of Trx, such as alkyl-2-imidazolyl disulfides and related compounds (e.g., 1 methylpropyl-2-imidazolyl disulfide) (Kirkpatrick et al., 1998 and 1999, supra) and naphthoquinone spiroketal derivatives (e.g., palmarumycin CP_1) (Wipf et al., 2001, supra); (2) specific inhibitors of TrxR, including gold complexes, such as aurothioglucose (ATG) and aurothiomalate (ATM) (see, e.g., the review by Gromer et al., 2004), which are examples of irreversible inhibitors of TrxR; (3) metal ions, such as Hg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+}, which can form readily complexes with thiols and selenols, and thus can be used in embodiments of the instant invention as inhibitors of TrxR or Trx; (4) inhibitors of G6PD, such as, for example, pyridoxal 5'-phosphate and 1 fluoro-2,4 dinitrobenzene (Milhausen and Levy 1975, supra), certain steroids, such as dehydroepiandrosterone (DHEA) and epiandrosterone (EA) (Gordon et al., 1995, supra); and (4) inhibitors of hexokinase activity (and thereby production of G6P for the G6PD), including chelators of metal ions, e.g. Mg^{2+}, such as EDTA, and compounds that react with SH groups, sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose,

2-C-hydroxy-methylglucose, xylose and lyxose (Sols et al., 1958, supra; McDonald, 1955, supra); further hexokinase inhibitors are disclosed in U.S. Pat. No. 5,854,067 entitled "Hexokinase Inhibitors." It will be understood that these inhibitors are listed for illustration only. Other Trx inhibitors exists and can be used, alone or in various combinations, in the methods of the present invention.

"Trx inhibitors" for use in the methods of the present invention also include reagents whereby the reduction of recombinantly produced antibodies or proteins may be reduced or prevented by decreasing the levels of enzymes of the Trx system, the pentose phosphate pathway or hexokinase at various points during the production campaign. In some embodiments, this reduction of enzyme levels may be accomplished by the use of targeted siRNAs, antisense nucleotides, or antibodies. To design targeted siRNAs or antisense nucleotides to the genes as found in CHO cells, these gene sequences are available from public databases to select sequences for targeting enzymes in different organisms. See Example 9 below for examples of the genes of the *E. coli* and mouse Trx system.

In addition to using inhibitors discussed above, it is also possible in certain embodiments of the instant invention to prevent the reduction of a recombinant protein to be purified by sparging the HCCF with air to maintain an oxidizing redox potential in the HCCF. This is a non-directed measure that can deplete glucose, G6P and NADPH by continuously oxidizing the reduced forms of Trx and TrxR. Air sparging of the HCCF tank can be performed, for example, with an air flow of about 100 liters to about 200 liters, such as, for example, 150 liters per minutes. Air sparging can be performed to reach an endpoint percentage of saturation; for example, air sparging can be continued until the HCCF is about 100% saturated with air, or it can be continued until the HCCR is about 30% saturated with air, or until it is between about 100% saturated to about 30% saturated with air. The minimum amount of dissolved oxygen (dO_2) required for the desired inhibitory effect also depends on the antibody or other recombinant protein produced. Thus, for example, about 10% dO_2 (or about 10 sccm for continuous stream) will have the desired effect during the production of antibody 2H7 (Variant A), while Apomab might require a higher (about 30%) dO_2.

In further embodiments of the instant invention, another non-directed method usable to block the reduction of the recombinant protein is lowering the pH of the HCCF. This embodiment takes advantage of particularly slow thiol-disulfide exchange at lower pH values (Whitesides et al., 1977, supra; Pleasants et al., 1989, supra). Therefore, the activity of the Trx system is significantly lower at pH values below 6, and thus the reduction of the recombinant protein, such as ocrelizumab, can be inhibited.

The non-directed approaches can also be combined with each other and/or with the use of one or more Trx inhibitors.

Disulfide bond reduction can be inhibited (i.e., partially or fully blocked) by using one or more Trx inhibitors and/or applying non-directed approaches following completion of the cell culture process, preferably to CCF prior to harvest or in the HCCF immediately after harvest. The optimal time and mode of application and effective amounts depend on the nature of the protein to be purified, the recombinant host cells, and the specific production method used. Determination of the optimal parameters is well within the skill of those of ordinary skill in the art.

For example, in a mammalian cell culture process, such as the CHO antibody production process described in the Examples herein, if cupric sulfate ($CuSO_4$ in the form of pentahydrate or the anhydrous form) is used as a Trx inhibitor, it can be added to supplement the CCF or HCCF in the concentration range of from about 5 μM to about 100 μM, such as from about 10 μM to about 80 μM, preferably from about 15 μM to about 50 μM. Since some cell cultures already contain copper (e.g. about 0.04 μM $CuSO_4$ for the CHO cell cultures used in the Examples herein), this amount is in addition to the copper, if any, already present in the cell culture. Any copper (II) salt can be used instead of $CuSO_4$ as long as solubility is not an issue. For example, copper acetate and copper chloride, which are both soluble in water, can be used instead of $CuSO_4$. The minimum effective concentration may also depend on the antibody produced and the stage where the inhibitor is used. Thus, for example, when cupric sulfate is added pre-lysis, for antibody 2H7 (Variant A) the minimum effective concentration is about 30 μM, for Apomab is about 75 μM, and for antibody Variant C (see Table 2) is about 50 μM. When cupric sulfate is added in CC medium, for antibody 2H7 (Variant A) the minimum effective concentration is about 15 μM, for Apomab is about 25 μM, and for antibody Variant C is about 20 μM. One typical minimal $CuSO_4$ inhibitor concentration of 2×Trx concentration (or Trx equivalence).

EDTA can be used in a wide concentration range, depending on the extent of cell lysis, the recombinant host cell used, and other parameters of the production process. For example, when using CHO or other mammalian host cells, EDTA can be typically added in a concentration of between about 5 mM to about 60 mM, such as from about 10 mM to about 50 mM, or from about 20 mM to about 40 mM, depending on the extent of cell lysis. For lower degree of cell lysis, lower concentrations of EDTA will suffice, while for a cell lysis of about 75%-100%, the required EDTA concentration is higher, such as, for example, from about 20 mM to about 40 mM. The minimum effective concentration may also depend on the antibody produced. Thus, for example, for antibody 2H7 (Variant A) the minimum effective EDTA concentration is about 10 mM.

DHEA as a Trx inhibitor is typically effective at a lower concentration, such as for example, in the concentration range from about 0.05 mM to about 5 mM, preferably from about 0.1 mM to about 2.5 mM.

Other Trx inhibitors, such as aurothioglucose (ATG) and aurothiomalate (ATM) inhibit reduction of disulfide bonds in the μM concentration range. Thus, for example, ATG or ATM may be added in a concentration between about 0.1 mM to about 1 mM. While the minimum inhibitory concentration varies depending on the actual conditions, for ATG and ATM typically it is around 4×TrxR concentration.

It is noted that all inhibitors can be used in an excess amount, therefore, it is not always necessary to know the amount of Trx or TrxR in the system.

In a preferred embodiment, the mammalian host cell used in the manufacturing process is a chinese hamster ovary (CHO) cell (Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)). Other mammalian host cells include, without limitation, monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture), Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary

tumor (MMT 060562, ATCC CCL51); TR1 cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; a human hepatoma line (Hep G2); and myeloma or lymphoma cells (e.g. Y0, J558L, P3 and NS0 cells) (see U.S. Pat. No. 5,807,715).

A preferred host cell for the production of the polypeptides herein is the CHO cell line DP12 (CHO K1 dhfr$^-$). This is one of the best known CHO cell lines, widely used in laboratory practice (see, for example, EP 0,307,247, published Mar. 15, 1989). In addition, other CHO-K1 (dhfr$^-$) cell lines are known and can be used in the methods of the present invention.

The mammalian host cells used to produce peptides, polypeptides and proteins can be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM, Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace (1979), Meth. in Enz. 58:44, Barnes and Sato (1980), Anal. Biochem. 102:255, U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. No. Re. 30,985; or U.S. Pat. No. 5,122,469, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

A protocol for the production, recovery and purification of recombinant antibodies in mammalian, such as CHO, cells may include the following steps:

Cells may be cultured in a stirred tank bioreactor system and a fed batch culture, procedure is employed. In a preferred fed batch culture the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed batch culture can include, for example, a semi-continuous fed batch culture, wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium. Fed batch culture is distinguished from simple batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed batch culture can be further distinguished from perfusion culturing insofar as the supernate is not removed from the culturing vessel during the process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers etc. and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cells of the culture may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, a single step or multiple step culture procedure may be employed. In a single step culture the host cells are inoculated into a culture environment and the processes are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture can be used. In the multi-stage culture cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

In certain embodiments, fed batch or continuous cell culture conditions may be devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In the growth phase cells are grown under conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (dO_2) and the like, are those used with the particular host and will be apparent to the ordinarily skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO_2) or a base (e.g., $Na2CO_3$ or NaOH). A suitable temperature range for culturing mammalian cells such as CHO cells is between about 30° C. to 38° C., and a suitable dO_2 is between 5-90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above the production phase or step may be continuous with the inoculation or growth phase or step.

The cell culture environment during the production phase of the cell culture is typically controlled. Thus, if a glycoprotein is produced, factors affecting cell specific productivity of the mammalian host cell may be manipulated such that the desired sialic acid content is achieved in the resulting glycoprotein. In a preferred aspect, the production phase of the cell culture process is preceded by a transition phase of the cell culture in which parameters for the production phase of the cell culture are engaged. Further details of this process are found in U.S. Pat. No. 5,721,121, and Chaderjian et al., *Biotechnol. Prog.* 21(2):550-3 (2005), the entire disclosures of which are expressly incorporated by reference herein.

Following fermentation proteins are purified. Procedures for purification of proteins from cell debris initially depend on the site of expression of the protein. Some proteins can be caused to be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For the latter proteins, the first step of a purification process involves lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. The same problem arises, although on a smaller scale, with directly secreted proteins due to the natural death of cells and release of intracellular host cell proteins and components in the course of the protein production run.

Once a clarified solution containing the protein of interest has been obtained, its separation from the other proteins produced by the cell is usually attempted using a combination of different chromatography techniques. These techniques separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size. Several different chromatography resins are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. The essence of each of these separation methods is that proteins can be caused either to move at different rates down a long column, achieving a

physical separation that increases as they pass further down the column, or to adhere selectively to the separation medium, being then differentially eluted by different solvents. In some cases, the desired protein is separated from impurities when the impurities specifically adhere to the column, and the protein of interest does not, that is, the protein of interest is present in the "flow-through." Thus, purification of recombinant proteins from the cell culture of mammalian host cells may include one or more affinity (e.g. protein A) and/or ion exchange chomarographic steps.

Ion exchange chromatography is a chromatographic technique that is commonly used for the purification of proteins. In ion exchange chromatography, charged patches on the surface of the solute are attracted by opposite charges attached to a chromatography matrix, provided the ionic strength of the surrounding buffer is low. Elution is generally achieved by increasing the ionic strength (i.e. conductivity) of the buffer to compete with the solute for the charged sites of the ion exchange matrix. Changing the pH and thereby altering the charge of the solute is another way to achieve elution of the solute. The change in conductivity or pH may be gradual (gradient elution) or stepwise (step elution). In the past, these changes have been progressive; i.e., the pH or conductivity is increased or decreased in a single direction.

For further details of the industrial purification of therapeutic antibodies see, for example, Fahrner et al., *Biotechnol. Genet. Eng. Rev.* 18:301-27 (2001), the entire disclosure of which is expressly incorporated by reference herein.

In addition to mammalian host cells, other eukaryotic organisms can be used as host cells for expression of the recombinant protein. For expression in yeast host cells, such as common baker's yeast or *Saccharomyces cerevisiae*, suitable vectors include episomally-replicating vectors based on the 2-micron plasmid, integration vectors, and yeast artificial chromosome (YAC) vectors. Other yeast suitable for recombinant production of heterologous proteins include *Schizosaccharomyces pombe* (Beach and Nurse, Nature, 290: 140 (1981); EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. Pat. No. 4,943,529; Fleer et al., Bio/Technology, 2: 968 975 (1991)) such as, e.g., *K. lactis* (MW98-8C, CBS683, CBS4574; Louvencourt et al., J. Bacteriol., 737 (1983)), *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906; Van den Berg et al., Bio/Technology, 8: 135 (1990)), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishna et al., J. Basic Microbiol., 28: 265 278 (1988)); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa* (Case et al., Proc. Natl. Acad. Sci. USA, 76: 5259 5263 (1979)); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31 Oct. 1990); and filamentous fungi such as, e.g., *Neurospora, Penicillium, Tolypocladium* (WO 91/00357 published 10 Jan. 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance et al., Biochem. Biophys. Res. Commun., 112: 284 289 (1983); Tilburn et al., Gene, 26: 205 221 (1983); Yelton et al., Proc. Natl. Acad. Sci. USA, 81: 1470 1474 (1984)) and *A. niger* (Kelly and Hynes, EMBO J., 4: 475 479 (1985)). Methylotropic yeasts are suitable herein and include, but are not limited to, yeast capable of growth on methanol selected from the genera consisting of *Hansenula, Candida, Kloeckera, Pichia, Saccharomyces, Torulopsis*, and *Rhodotorula*. A list of specific species that are exemplary of this class of yeasts may be found in C. Anthony, The Biochemistry of Methylotrophs, 269 (1982). Expression systems for the listed and other yeasts are well known in the art and/or are commercially available.

For expression in insect host cells, such as Sf9 cells, suitable vectors include baculoviral vectors. For expression in plant host cells, particularly dicotyledonous plant hosts, such as tobacco, suitable expression vectors include vectors derived from the Ti plasmid of *Agrobacterium tumefaciens.*

The methods of the present invention also extend to cultures of prokaryotic host cells. Prokaryotic host cells suitable for expressing antibodies and other proteins to be protected by means of the instant invention include Archaebacteria and Eubacteria, such as Gram-negative or Gram-positive organisms. Examples of useful bacteria include *Escherichia* (e.g., *E. coli*), *Bacilli* (e.g., *B. subtilis*), *Enterobacteria, Pseudomonas* species (e.g., *P. aeruginosa*), *Salmonella typhimurium, Serratia marcescans, Klebsiella, Proteus, Shigella, Rhizobia, Vitreoscilla*, or *Paracoccus*. In one embodiment, gram-negative cells are used. Examples of *E. coli* strains include strain W3110 (Bachmann, Cellular and Molecular Biology, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC Deposit No. 27,325) and derivatives thereof, including strain 33D3 having genotype W3110 ΔfhuA (ΔtonA) ptr3 lac Iq lacL8 ΔompTΔ(nmpc-fepE) degP41 kanR (U.S. Pat. No. 5,639,635). Other strains and derivatives thereof, such as *E. coli* 294 (ATCC 31,446), *E. coli* B, *E. coli* 1776 (ATCC 31,537) and *E. coli* RV308 (ATCC 31,608) are also suitable. These examples are illustrative rather than limiting. Methods for constructing derivatives of any of the above-mentioned bacteria having defined genotypes are known in the art and described in, for example, Bass et al., Proteins, 8:309-314 (1990). It is generally necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, *E. coli, Serratia*, or *Salmonella* species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon. Typically the host cell should secrete minimal amounts of proteolytic enzymes, and additional protease inhibitors may desirably be incorporated in the cell culture.

Methods for the production, recovery and purification of recombinant proteins from non-mammalian host cell cultures are also well known in the art. If the polypeptide is produced in a non-mammalian cell, e.g., a microorganism such as fungi or *E. coli*, the polypeptide will be recovered inside the cell or in the periplasmic space (Kipriyanov and Little, *Molecular Biotechnology,* 12: 173 201 (1999); Skerra and Pluckthun, *Science,* 240: 1038 1040 (1988)). Hence, it is necessary to release the protein from the cells to the extracellular medium by extraction such as cell lysis. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration.

Cell lysis is typically accomplished using mechanical disruption techniques such as homogenization or head milling. While the protein of interest is generally effectively liberated, such techniques have several disadvantages (Engler, *Protein Purification Process Engineering*, Harrison eds., 37 55 (1994)). Temperature increases, which often occur during processing, may result in inactivation of the protein. Moreover, the resulting suspension contains a broad spectrum of contaminating proteins, nucleic acids, and polysaccharides. Nucleic acids and polysaccharides increase solution viscosity, potentially complicating subsequent processing by centrifugation, cross-flow filtration, or chromatography. Complex associations of these contaminants with the protein of interest can complicate the purification process and result in unacceptably low yields. Improved methods for purification of heterologous polypeptides from microbial fermentation

broth or homogenate are described, for example, in U.S. Pat. No. 7,169,908, the entire disclosure of which is expressly incorporated herein by reference.

It is emphasized that the fermentation, recovery and purification methods described herein are only for illustration purposes. The methods of the present invention can be combined with any manufacturing process developed for the production, recovery and purification of recombinant proteins.

2. Antibodies

In a preferred embodiment, the methods of the present invention are used to prevent the reduction of inter- and/or intrachain disulfide bonds of antibodies, including therapeutic and diagnostic antibodies. Antibodies within the scope of the present invention include, but are not limited to: anti-HER2 antibodies including Trastuzumab (HERCEPTIN®) (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285-4289 (1992), U.S. Pat. No. 5,725,856); anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN®), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108B1, or Tositumomab (BEXXAR®); anti-IL-8 (St John et al., *Chest,* 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors,* 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., *Transplant Intl.* 4:3-7 (1991), and Hourmant et al., *Transplantation* 58:377-380 (1994)); anti-IgE (Presta et al., *J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul. 31, 1997); anti-IgE (including E25, E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No. 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No. PCT/US98/13410 filed Jun. 30, 1998, U.S. Pat. No. 5,714,338); anti-Apo-2 receptor antibody (WO 98/51793 published Nov. 19, 1998); anti-TNF-α antibodies including cA2 (REMICADE®), CDP571 and MAK-195 (See, U.S. Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al., *J. Immunol.* 156(4):1646-1653 (1996), and Dhainaut et al., *Crit. Care Med.* 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human $\alpha_4\beta_7$ integrin (WO 98/06248 published Feb. 19, 1998); anti-EGFR (chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S. Pat. No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-tac antibodies such as CHI-621 (SIMULECT®) and (ZENAPAX®) (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997); anti-CD4 antibodies such as the cM-7412 antibody (Choy et al., *Arthritis Rheum* 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al., *Nature* 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22 antibody directed against FcγRI as in Graziano et al., *J. Immunol.* 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al., *Cancer Res.* 55(23 Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al., *Cancer Res.* 55(23): 5852s-5856s (1995); and Richman et al., *Cancer Res.* 55(23 Supp): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al., *Eur J. Immunol.* 26(1):1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al., *J. Immunol.* 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al., *Cancer Res* 55(23 Suppl): 5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22 antibodies such as LL2 or LymphoCide (Juweid et al., *Cancer Res* 55(23 Suppl): 5899s-5907s (1995)); anti-EpCAM antibodies such as 17-1A (PANOREX®); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO®); anti-RSV antibodies such as MEDI-493 (SYNAGIS®); anti-CMV antibodies such as PROTOVIR®; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR®; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-αvβ3 antibody VITAXIN®; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1). The preferred target antigens for the antibody herein are: HER2 receptor, VEGF, IgE, CD20, CD11a, and CD40.

Many of these antibodies are widely used in clinical practice to treat various diseases, including cancer.

In certain specific embodiments, the methods of the present invention are used for the production of the following antibodies and recombinant proteins.

Anti-CD20 Antibodies

Rituximab (RITUXAN®) is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. Rituximab is the antibody called "C2B8" in U.S. Pat. No. 5,736,137 issued Apr. 7, 1998 (Anderson et al.). Rituximab is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20-positive, B cell non-Hodgkin's lymphoma. In vitro mechanism of action studies have demonstrated that rituximab binds human complement and lyses lymphoid B cell lines through complement-dependent cytotoxicity (CDC) (Reff et al., *Blood* 83(2):435-445 (1994)). Additionally, it has significant activity in assays for antibody-dependent cellular cytotoxicity (ADCC). More recently, rituximab has been shown to have anti-proliferative effects in tritiated thymidine incorporation assays and to induce apoptosis directly, while other anti-CD19 and CD20 antibodies do not (Maloney et al., *Blood* 88(10):637a (1996)). Synergy between rituximab and chemotherapies and toxins has also been observed experimentally. In particular, rituximab. sensitizes drug-resistant human B cell lymphoma cell lines to the cytotoxic effects of doxorubicin, CDDP, VP-1 6, diphtheria toxin and ricin (Demidem et al., *Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997)). In vivo preclinical studies have shown that rituximab depletes B cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff et al., *Blood* 83(2):435-445 (1994)).

Patents and patent publications concerning CD20 antibodies include U.S. Pat. Nos. 5,776,456, 5,736,137, 6,399,061, and 5,843,439, as well as U.S. patent application Nos. US 2002/0197255A1, US 2003/0021781A1, US 2003/0082172 A1, US 2003/0095963 A1, US 2003/0147885 A1 (Anderson et al.); U.S. Pat. No. 6,455,043B1 and WO00/09160 (Grillo-Lopez, A.); WO00/27428 (Grillo-Lopez and White); WO00/27433 (Grillo-Lopez and Leonard); WO00/44788 (Braslawsky et al.); WO01/10462 (Rastetter, W.); WO01/10461 (Rastetter and White); WO01/10460 (White and Grillo-Lopez); U.S. application No. US2002/0006404 and WO02/04021 (Hanna and Hariharan); U.S. application No. US2002/0012665 A1 and WO01/74388 (Hanna, N.); U.S. application

No. US 2002/0058029 A1 (Hanna, N.); U.S. application No. US 2003/0103971 A1 (Hariharan and Hanna); U.S. application No. US2002/0009444A1, and WO01/80884 (Grillo-Lopez, A.); WO01/97858 (White, C.); U.S. application No. US2002/0128488A1 and WO02/34790 (Reff, M.); W) 02/060955 (Braslawsky et al.); WO2/096948 (Braslawsky et al.); WO02/079255 (Reff and Davies); U.S. Pat. No. 6,171, 586B1, and WO98/56418 (Lam et al.); WO98/58964 (Raju, S.); WO99/22764 (Raju, S.); WO99/51642, U.S. Pat. No. 6,194,551B1, U.S. Pat. No. 6,242,195B1, U.S. Pat. No. 6,528,624B1 and U.S. Pat. No. 6,538,124 (Idusogie et al.); WO00/42072 (Presta, L.); WO00/67796 (Curd et al.); WO01/03734 (Grillo-Lopez et al.); U.S. application No. US 2002/0004587A1 and WO01/77342 (Miller and Presta); U.S. application No. US2002/0197256 (Grewal, I.); U.S. application No. US 2003/0157108 A1 (Presta, L.); U.S. Pat. Nos. 6,090,365B1, 6,287,537B1, 6,015,542, 5,843,398, and 5,595,721, (Kaminski et al.); U.S. Pat. Nos. 5,500,362, 5,677, 180, 5,721,108, and 6,120,767 (Robinson et al.); U.S. Pat. No. 6,410,391B1 (Raubitschek et al.); U.S. Pat. No. 6,224, 866B1 and WO00/20864 (Barbera-Guillem, E.); WO01/13945 (Barbera-Guillem, E.); WO00/67795 (Goldenberg); U.S. application No. US 2003/01339301 A1 and WO00/74718 (Goldenberg and Hansen); WO00/76542 (Golay et al.); WO01/72333 (Wolin and Rosenblatt); U.S. Pat. No. 6,368,596B1 (Ghetie et al.); U.S. application No. US2002/0041847 A1, (Goldenberg, D.); U.S. application No. US2003/0026801A1 (Weiner and Hartmann); WO02/102312 (Engleman, E.); U.S. patent application No. 2003/0068664 (Albitar et al.); WO03/002607 (Leung, S.); WO 03/049694 and US 2003/0185796 A1 (Wolin et al.); WO03/061694 (Sing and Siegall); US 2003/0219818 A1 (Bohen et al.); US 2003/0219433 A1 and WO 03/068821 (Hansen et al.) each of which is expressly incorporated herein by reference. See, also, U.S. Pat. No. 5,849,898 and EP application no. 330,191 (Seed et al.); U.S. Pat. No. 4,861,579 and EP332, 865A2 (Meyer and Weiss); U.S. Pat. No. 4,861,579 (Meyer et al.) and WO95/03770 (Bhat et al.).

Publications concerning therapy with Rituximab include: Perotta and Abuel "Response of chronic relapsing ITP of 10 years duration to Rituximab" Abstract #3360 *Blood* 10(1) (part 1-2): p. 88B (1998); Stashi et al., "Rituximab chimeric anti-CD20 monoclonal antibody treatment for adults with chronic idiopathic thrombocytopenic purpura" *Blood* 98(4): 952-957 (2001); Matthews, R. "Medical Heretics" New Scientist (7 Apr. 2001); Leandro et al., "Clinical outcome in 22 patients with rheumatoid arthritis treated with B lymphocyte depletion" *Ann Rheum Dis* 61:833-888 (2002); Leandro et al., "Lymphocyte depletion in rheumatoid arthritis: early evidence for safety, efficacy and dose response. *Arthritis & Rheumatism* 44(9): 5370 (2001); Leandro et al., "An open study of B lymphocyte depletion in systemic lupus erythematosus", *Arthritis & Rheumatism* 46(1):2673-2677 (2002); Edwards and Cambridge "Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes" *Rheumatology* 40:205-211 (2001); Edwards et al., "B-lymphocyte depletion therapy in rheumatoid arthritis and other autoimmune disorders" *Biochem. Soc. Trans.*

30(4):824-828 (2002); Edwards et al., "Efficacy and safety of Rituximab, a B-cell targeted chimeric monoclonal antibody: A randomized, placebo controlled trial in patients with rheumatoid arthritis. *Arthritis & Rheumatism* 46(9): S197 (2002); Levine and Pestronk "IgM antibody-related polyneuropathies: B-cell depletion chemotherapy using Rituximab" *Neurology* 52: 1701-1704 (1999); DeVita et al., "Efficacy of selective B cell blockade in the treatment of rheumatoid arthritis" *Arthritis & Rheumatism* 46:2029-2033 (2002); Hidashida et al., "Treatment of DMARD-Refractory rheumatoid arthritis with rituximab." Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002; Tuscan, J. "Successful treatment of Infliximab-refractory rheumatoid arthritis with rituximab" Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002. Sarwal et al., *N. Eng. J. Med.* 349(2):125-138 (Jul. 10, 2003) reports molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling.

In various embodiments, the invention provides pharmaceutical compositions comprising humanized 2H7 anti-CD20 antibodies. In specific embodiments, the humanized 2H7 antibody is an antibody listed in Table 2.

TABLE 2

Humanized anti-CD20 Antibody and Variants Thereof

2H7 variant	V_L SEQ ID NO.	V_H SEQ ID NO.	Full L chain SEQ ID NO.	Full H chain SEQ ID NO.
A	1	2	6	7
B	1	2	6	8
C	3	4	9	10
D	3	4	9	11
F	3	4	9	12
G	3	4	9	13
H	3	5	9	14
I	1	2	6	15

Each of the antibody variants A, B and I of Table 2 comprises the light chain variable sequence (V_L):

(SEQ ID NO: 1)
DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHWYQQKPGKAPKPLIYA

PSNLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWSFNPPTFG

QGTKVEIKR;
and

the heavy chain variable sequence (V_H):

(SEQ ID NO: 2)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA

IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV

YYSNSYWYFDVWGQGTLVTVSS.

Each of the antibody variants C, D, F and G of Table 2 comprises the light chain variable sequence (V_L):

(SEQ ID NO: 3)
DIQMTQSPSSLSASVGDRVTITCRASSSVSYLHWYQQKPGKAPKPLIYAP

SNLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWAFNPPTFGQG

TKVEIKR,
and

the heavy chain variable sequence (V_H):

```
                                              (SEQ ID NO: 4)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSASYWYFDVWGQGTLVTVSS.
```

The antibody variant H of Table 2 comprises the light chain variable sequence (V_L) of SEQ ID NO:3 (above) and the heavy chain variable sequence (V_H):

```
                                              (SEQ ID NO: 5)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSYRYWYFDVWGQGTLVTVSS
```

Each of the antibody variants A, B and I of Table 2 comprises the full length light chain sequence:

```
                                              (SEQ ID NO: 6)
DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHWYQQKPGKAPKPLIYAP
SNLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWSFNPPTFGQG
TKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD
NALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL
SSPVTKSFNRGEC.
```

Variant A of Table 2 comprises the full length heavy chain sequence:

```
                                              (SEQ ID NO: 7)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSNSYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.
```

Variant B of Table 2 comprises the full length heavy chain sequence:

```
                                              (SEQ ID NO: 8)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSNSYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
```

-continued

```
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.
```

Variant I of Table 2 comprises the full length heavy chain sequence:

```
                                              (SEQ ID NO: 15)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSNSYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNAALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.
```

Each of the antibody variants C. D, F, G and H of Table 2 comprises the full length light chain sequence:

```
                                              (SEQ ID NO: 9)
DIQMTQSPSSLSASVGDRVTITCRASSSVSYLHWYQQKPGKAPKPLIYAP
SNLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWAFNPPTFGQG
TKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD
NALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL
SSPVTKSFNRGEC.
```

Variant C of Table 2 comprises the full length heavy chain sequence:

```
                                              (SEQ ID NO: 10)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSASYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.
```

Variant D of Table 2 comprises the full length heavy chain sequence:

```
                                           (SEQ ID NO: 11)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA

IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV

YYSASYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL

VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT

QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP

KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ

YNATYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEATISKAKGQPRE

PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP

PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP

GK.
```

Variant F of Table 2 comprises the full length heavy chain sequence:

```
                                           (SEQ ID NO: 12)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA

IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV

YYSASYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL

VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT

QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP

KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ

YNATYRVVSVLTVLHQDWLNGKEYKCKVSNAALPAPIAATISKAKGQPRE

PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP

PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP

GK.
```

Variant G of Table 2 comprises the full length heavy chain sequence:

```
                                           (SEQ ID NO: 13)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA

IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV

YYSASYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL

VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT

QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP

KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ

YNATYRVVSVLTVLHQDWLNGKEYKCKVSNAALPAPIAATISKAKGQPRE

PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP

PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHWHYTQKSLSLSP

GK.
```

Variant H of Table 2 comprises the full length heavy chain sequence:

```
                                           (SEQ ID NO: 14)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA

IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV

YYSYRYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL

VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT

QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP

KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ

YNATYRVVSVLTVLHQDWLNGKEYKCKVSNAALPAPIAATISKAKGQPRE

PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP

PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP

GK.
```

In certain embodiments, the humanized 2H7 antibody of the invention further comprises amino acid alterations in the IgG Fc and exhibits increased binding affinity for human FcRn over an antibody having wild-type IgG Fc, by at least 60 fold, at least 70 fold, at least 80 fold, more preferably at least 100 fold, preferably at least 125 fold, even more preferably at least 150 fold to about 170 fold.

The N-glycosylation site in IgG is at Asn297 in the C_H2 domain. Humanized 2H7 antibody compositions of the present invention include compositions of any of the preceding humanized 2H7 antibodies having a Fc region, wherein about 80-100% (and preferably about 90-99%) of the antibody in the composition comprises a mature core carbohydrate structure which lacks fucose, attached to the Fc region of the glycoprotein. Such compositions were demonstrated herein to exhibit a surprising improvement in binding to FcγRIIIA(F158), which is not as effective as FcγRIIIA (V158) in interacting with human IgG. FcγRIIIA (F158) is more common than FcγRIIIA (V158) in normal, healthy African Americans and Caucasians. See Lehrnbecher et al., *Blood* 94:4220 (1999). Historically, antibodies produced in Chinese Hamster Ovary Cells (CHO), one of the most commonly used industrial hosts, contain about 2 to 6% in the population that are nonfucosylated. YB2/0 and Lec13, however, can produce antibodies with 78 to 98% nonfucosylated species. Shinkawa et al., *J Bio. Chem.* 278 (5), 3466-347 (2003), reported that antibodies produced in YB2/0 and Lec13 cells, which have less FUT8 activity, show significantly increased ADCC activity in vitro. The production of antibodies with reduced fucose content are also described in e.g., Li et al., (GlycoFi) "Optimization of humanized IgGs in glycoengineered *Pichia pastoris*" in Nature Biology online publication 22 Jan. 2006; Niwa R. et al., *Cancer Res.* 64(6):2127-2133 (2004); US 2003/0157108 (Presta); U.S. Pat. No. 6,602,684 and US 2003/0175884 (Glycart Biotechnology); US 2004/0093621, US 2004/0110704, US 2004/0132140 (all of Kyowa Hakko Kogyo).

A bispecific humanized 2H7 antibody encompasses an antibody wherein one arm of the antibody has at least the antigen binding region of the H and/or L chain of a humanized 2H7 in antibody of the invention, and the other arm has V region binding specificity for a second antigen. In specific embodiments, the second antigen is selected from the group consisting of CD3, CD64, CD32A, CD16, NKG2D or other NK activating ligands.

Anti-HER2 Antibodies

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, trastuzumab or HERCEPTIN®; U.S. Pat. No. 5,821,337) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). Trastuzumab received marketing approval from the Food and Drug Administration (FDA) Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors over-express the HER2 protein. In November 2006, the FDA approved Herceptin as part of a treatment regimen containing doxorubicin, cyclophosphamide and paclitaxel, for the adjuvant treatment of patients with HER2-positive, node-positive breast cancer.

In one embodiment, the anti-HER2 antibody comprises the following V_L and V_H domain sequences:

humanized 2C4 version 574 antibody V_L (SEQ ID NO: 16)

DIQMTQSPSSLSASVGDRVTITC**KASQDVSIGVA**WYQQKPGKAPKLLIYS

ASYRYTGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC**QQYYIYPYT**FGQ

GTKVEIK.

and humanized 2C4 version 574 antibody V_H (SEQ ID NO: 17)

EVQLVESGGGLVQPGGSLRLSCAAS**GFTFTDYTMD**WVRQAPGKGLEWVA**D**

VNPNSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARN**L**

GPSFYFDYWGQGTLVTVSS.

In another embodiment, the anti-HER2 antibody comprises the V_L (SEQ ID NO:18) and V_H (SEQ ID NO:19) domain sequences of trastuzumab as shown in FIG. **21** and FIG. **22**, respectively.

Other HER2 antibodies with various properties have been described in Tagliabue et al., *Int. J. Cancer* 47:933-937 (1991); McKenzie et al., *Oncogene* 4:543-548 (1989); Maier et al., *Cancer Res.* 51:5361-5369 (1991); Bacus et al., *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al., *PNAS* (*USA*) 88:8691-8695 (1991); Bacus et al., *Cancer Research* 52:2580-2589 (1992); Xu et al., *Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk et al., *Cancer Research* 52:2771-2776 (1992); Hancock et al., *Cancer Res.* 51:4575-4580 (1991); Shawver et al., *Cancer Res.* 54:1367-1373 (1994); Arteaga et al., *Cancer Res.* 54:3758-3765 (1994); Harwerth et al., *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; and Klapper et al., *Oncogene* 14:2099-2109 (1997).

Anti-VEGF Antibodies

The anti-VEGF antibodies may, for example, comprise the following sequences:

In one embodiment, the anti-VEGF antibody comprises the following V_L sequence (SEQ ID NO:20):

DIQMTQTTSS LSASLGDRVI ISCSASQDIS NYLNWYQQKP

DGTVKVLIYF TSSLHSGVPS RFSGSGSGTD YSLTISNLEP

EDIATYYCQQ YSTVPWTFGG GTKLEIKR;
and

the following V_H sequence (SEQ ID NO:21):

EIQLVQSGPE LKQPGETVRI SCKASGYTFT NYGMNWVKQA

PGKGLKWMGW INTYTGEPTY AADFKRRFTF SLETSASTAY

LQISNLKNDD TATYFCAKYP HYYGSSHWYF DVWGAGTTVT VSS.

In another embodiment, the anti-VEGF antibody comprises the following V_L sequence (SEQ ID NO:22):

DIQMTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP

GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP

EDFATYYCQQ YSTVPWTFGQ GTKVEIKR;
and

the following V_H sequence (SEQ ID NO:23):

EVQLVESGGG LVQPGGSLRL SCAASGYTFT NYGMNWVRQA

PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY

LQMNSLRAED TAVYYCAKYP HYYGSSHWYF DVWGQGTLVT VSS.

In a third embodiment, the anti-VEGF antibody comprises the following V_L sequence (SEQ ID NO:24):

DIQLTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP

GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP

EDFATYYCQQ YSTVPWTFGQ GTKVEIKR;
and

the following V_H sequence (SEQ ID NO:25):

EVQLVESGGG LVQPGGSLRL SCAASGYDFT HYGMNWVRQA

PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY

LQMNSLRAED TAVYYCAKYP YYYGTSHWYF DVWGQGTLVT VSS.

Anti-CD11a Antibodies

The humanized anti-CD11a antibody efalizumab or Raptiva® (U.S. Pat. No. 6,037,454) received marketing approval from the Food and Drug Administration on Oct. 27, 2003 for the treatment for the treatment of psoriasis. One embodiment provides for an anti-human CD11a antibody comprising the V_L and V_H sequences of HuMHM24 below:

V_L (SEQ ID NO: 26):
DIQMTQSPSSLSASVGDRVTITCRASKTISKYLAWYQQKPGKAPKLLIYS

GSTLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQHNEYPLTFGQ

GTKVEIKR;
and

V_H (SEQ ID NO: 27):
EVQLVESGGGLVQPGGSLRLSCAASGYSFTGHWMNWVRQAPGKGLEWVGM

IHPSDSETRYNQKFKDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARGI

YFYGTTYFDYWGQGTLVTVSS.

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The anti-human CD11a antibody may comprise the V_H of SEQ ID NO:27 and the full length L chain of HuMHM24 having the sequence of:

```
(SEQ ID NO: 28)
DIQMTQSPSSLSASVGDRVTITCRASKTISKYLAWYQQKPGKAPKLLIYS

GSTLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQHNEYPLTFGQ

GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV

DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG

LSSPVTKSFNRGEC,
or
```

the L chain above with the H chain having the sequence of:

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(SEQ ID NO: 29)
EVQLVESGGGLVQPGGSLRLSCAASGYSFTGHWMNWVRQAPGKGLEWVGM

IHPSDSETRYNQKFKDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARGI

YFYGTTYFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV

KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ

TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK

PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY

NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP

QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP

VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

K.
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Antibodies to the DR5 receptor (anti-DR5) antibodies can also be produced in accordance with the present invention. Such anti-DR5 antibodies specifically include all antibody variants disclosed in PCT Publication No. WO 2006/083971, such as the anti-DR5 antibodies designated Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3, especially Apomab 8.3 and Apomab 7.3, preferably Apomab 7.3. The entire content of WO 2006/083971 is hereby expressly incorporated by reference.

3. Other Disulfide-Containing Proteins

In addition to antibodies, the methods of the present invention find utility in the manufacturing of other polypeptides including disulfide bonds. Representative examples of such polypeptides include, without limitation, the following therapeutic proteins: tissue plasminogen activators (t-PAs), such as human tissue plasminogen activator (htPA, alteplase, ACTIVASE®), a thrombolytic agent for the treatment of myocardial infarction; a TNKase™, a ht-PA variant with extended half-life and fibrin specificity for single-bolus administration; recombinant human growth hormone (rhGH, somatropin, NUTROPIN®, PROTROPIN®) for the treatment of growth hormone deficiency in children and adults; and recombinant human deoxyribonuclease I (DNase I) for the treatment of cystic fibrosis (CF).

Examples of disulfide-containing biologically important proteins include growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of any of the above-listed polypeptides.

4. General Methods for the Recombinant Production of Antibodies

The antibodies and other recombinant proteins herein can be produced by well known techniques of recombinant DNA technology. Thus, aside from the antibodies specifically identified above, the skilled practitioner could generate antibodies directed against an antigen of interest, e.g., using the techniques described below.

Antigen Selection and Preparation

The antibody herein is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those proteins described in section (3) below. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20, CD22, CD34, CD40; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150, 95, VLA-4, ICAM-1, VCAM and αv/133 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group

antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C, or any of the other antigens mentioned herein. Antigens to which the antibodies listed above bind are specifically included within the scope herein.

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of antigen or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal Antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, Protein A-Sepharose, hydroxyapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. Preferably the Protein A chromatography procedure described herein is used.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl. Acad. Sci. USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

In a further embodiment, monoclonal antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional hybridoma techniques for isolation of monoclonal antibodies.

Humanized and Human Antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human FR for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immunol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production.

Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and Duchosal et al., *Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 (1991); Vaughan et al., *Nature Biotech* 14:309 (1996)).

Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv) (see WO 93/16185).

Multispecific Antibodies

Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first

antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994). Alternatively, the antibodies can be "linear antibodies" as described in Zapata et al., *Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

Immunoadhesins

The simplest and most straightforward immunoadhesin design combines the binding domain(s) of the adhesin (e.g. the extracellular domain (ECD) of a receptor) with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the immunoadhesins of the present invention, nucleic acid encoding the binding domain of the adhesin will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, C_H2 and C_H3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the C_H1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics of the immunoadhesin.

In a preferred embodiment, the adhesin sequence is fused to the N-terminus of the Fc domain of immunoglobulin G_1 (IgG_1). It is possible to fuse the entire heavy chain constant region to the adhesin sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (i.e. residue 216, taking the first residue of heavy chain constant region to be 114), or analogous sites of other immunoglobulins is used in the fusion. In a particularly preferred embodiment, the adhesin amino acid sequence is fused to (a) the hinge region and C_H2 and C_H3 or (b) the C_H1, hinge, C_H2 and C_H3 domains, of an IgG heavy chain.

For bispecific immunoadhesins, the immunoadhesins are assembled as multimers, and particularly as heterodimers or heterotetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of four basic units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each of the four units may be the same or different.

Various exemplary assembled immunoadhesins within the scope herein are schematically diagrammed below:

AC_L-AC_L;

AC_H-(AC_H, AC_L-AC_H, AC_L-V_HC_H, or V_LC_L-AC_H);

AC_L-AC_H-(AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H)

AC_L-V_HC_H-(AC_H, or AC_L-V_HC_H, or V_LC_L-AC_H);

V_LC_L-AC_H-(AC_L-V_HC_H, or V_LC_L-AC_H); and

$(A\text{-}Y)_n$-$(V_LC_L$-$V_HC_H)_2$,

wherein each A represents identical or different adhesin amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_H is an immunoglobulin heavy chain constant domain;

n is an integer greater than 1;

Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed to be present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the adhesin sequences can be inserted between immunoglobulin heavy chain and light chain sequences, such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the adhesin sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the C_H2 domain, or between the C_H2 and C_H3 domains. Similar constructs have been reported by Hoogenboom, et al., *Mol. Immunol.* 28:1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an adhesin-immunoglobulin heavy chain fusion polypeptide, or directly fused to the adhesin. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the adhesin-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Pat. No. 4,816,567, issued 28 Mar. 1989.

Immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the adhesin portion in-frame to an immunoglobulin cDNA sequence. However, fusion to genomic immunoglobulin fragments can also be used (see, e.g. Aruffo et al., *Cell* 61:1303-1313 (1990); and Stamenkovic et al., Cell 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequences from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the "adhesin" and the immunoglobulin parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Va.

Example 1

Description of Materials and Methods

The following materials and methods were used in Examples 2-8 below.

Materials

Materials and devices used in the experiments described in the experimental examples include: stainless steel vials (mini-tanks, Flow Components, Dublin, Calif.; short (50 cc) and tall (55 cc)); dialysis tubing (Spectra/Por, 6-8000 MWCO, cat. #132645), 0.22 μm filter (Millipore Millipak Gamma Gold cat. # MPGL04GH2); phosphate buffered saline (PBS, EMD, cat. #6506); ethylenediaminetetraacetic acid (EDTA, Sigma, cat. # E4884); α-nicotinamide adenine dinucleotide phosphate (NADPH, Calbiochem, cat. #481973); dehydroepiandrosterone (DHEA, TCI, cat. # D0044); cupric sulfate (Sigma, cat. # C8027), glucose-6-phosphate (G6P, Calbiochem, cat. #346764); aurothioglucose (ATG, USP, cat. #1045508); aurothiomalate (ATM, Alfa Aesar, cat. #39740); reduced glutathione (GSH, J. T. Baker, cat. # M770-01); monobromobimane (mBB, Fluka, cat. #69898); histidine (J. T. Baker, cat. #2080-05); sodium sulfate (J. T. Baker, cat. #3897-05); Trx (Sigma, cat. # T8690); TrxR (Sigma, cat. # T9698). All chemicals and reagents were used as received with no further purification. Stock solutions of EDTA (250 mM, pH 7.5), $CuSO_4$ (10 mM), ATG (30 mM), ATM (30 mM), NADPH (75 mM), G6P (300 mM) were prepared for use in the mini-tank time course studies.

Generation of Cell Culture Fluid (CCF)

In order to generate ocrelizumab CCF for the various reduction studies, a representative small-scale fermentation process was utilized similar to the methods described previously (Chaderjian et al., 2005). Briefly, 3 liter glass stirred-tank Applikon® bioreactors fitted with pitched blade impellers were used for the inoculum-train and production cultures with the ocrelizumab media components. The bioreactors were outfitted with calibrated dissolved oxygen (DO), pH and temperature probes. DO, pH, temperature, and agitation rate were controlled using digital control units to the defined parameters of the ocrelizumab manufacturing process. The working volume for both the inoculum-train and production cultures was 1.5 L. Daily samples were analyzed on a NOVA Bioprofile blood gas analyzer to ensure the accuracy of the on-line value for pH and dissolved oxygen as well as to monitor the glucose, lactate, ammonium, glutamine, glutamate, and sodium concentrations in the cultures. Daily samples were also taken to monitor cell growth, viability, and titer. Cell growth was measured both by viable cell counts using a ViCell as well as on a packed cell volume (PCV) basis. Culture viability was determined by trypan blue exclusion on a ViCell instrument. Supernatant samples were assayed by an HPLC-based method to measure ocrelizumab titer values.

Harvested Cell Culture Fluid (HCCF) Preparation

Complete lysis of CCF was achieved by high pressure homogenization using a Microfluidics HC-8000 homogenizer. The pressure regulator of the instrument was set to 4,000-8,000 psi, and the CCF was pulled in through the homogenizer to obtain complete cell lysis (membrane breakage) after a single pass. The CCF homogenate was collected once water was purged through the system. The homogenate was transferred to centrifuge bottles and centrifuged in a Sorval RC-3B rotor centrifuge at 4,500 rpm for 30 minutes at 20° C. The centrate was decanted and then depth filtered followed by 0.22 μm sterile filtration using a peristaltic pump with silicon tubing to generate the final HCCF from the homogenized CCF (100% cell lysis). Alternatively, the CCF

was centrifuged straight from the fermentor without any homogenization and then the centrate was filtered with a sterile 0.22 μm filter to generate the HCCF.

Mini-Tank Handling

A laminar flow hood was used in handling all mini-tanks and all materials used in the HCCF incubation experiments were either autoclaved or rinsed using 70% isopropanol to minimize bacterial contamination.

Lactate Dehydrogenase Assay

For lactate dehydrogenase assay, see Babson & Babson (1973) and Legrand et al., (1992), which are hereby incorporated by reference.

Dialysis Experiment

A dialysis experiment was carried out in order to determine whether the components causing reduction of ocrelizumab were small molecules or macromolecules (i.e. enzymes). A sample of 3 mL of purified and formulated ocrelizumab (30.2 mg/mL) was dialyzed against 1 L of phosphate buffered saline (PBS, 10 mM pH 7.2) for 24 hours and the PBS was changed after 8 hours. The concentration of the ocrelizumab sample was then adjusted to 1 mg/mL using the absorbance at 280 nm. Aliquots were stored at −70° C. prior to use. Dialysis tubing was hydrated overnight in a 0.05% azide solution and rinsed with sterile water prior to use. The HCCF obtained from homogenization of CCF from a 3-L fermentor was thawed and filtered through a 0.22 μm Millipak filter using a peristaltic pump. Six short mini-tanks were filled with 30 mL of HCCF each. To each mini-tank, 500 μL of ocrelizumab sample in sealed dialysis tubing was added. The mini-tanks were sealed and loaded into a bench top mixer (Barnstead Lab-Line MAX Q 4000) operating at 35 rpm and ambient temperature. For each time-point, one mini-tank was removed from the mixer, and aliquots of the HCCF (in the mini-tank) and ocrelizumab sample (in the dialysis bag) were taken and stored at −70° C. until analyzed with the free thiol assay and the Bioanalyzer assay (described below).

Test Inhibitors for Reduction in a Small-Scale In Vitro System

A tall mini-tank was filled with 27 mL of HCCF. Depending on the experiment design, various reagents (NADPH, G6P, inhibitors of G6PD or TrxR) were added to the desired concentration, and the final volume in the mini-tank was brought to 30 mL with PBS (10 mM pH 7.2). The mini-tanks were sealed and loaded into a bench top mixer running at 35 rpm and ambient temperature. At each-time point for sampling, the exteriors of the mini-tanks were sterilized with 70% IPA and opened in a laminar flow hood for the removal of an aliquot. The mini-tanks were then re-sealed and loaded back into the bench top mixer. All aliquots were stored at −70° C. until analyzed with the free thiol assay and Bioanalyzer assay (described below).

In Vitro Trx/TrxReductase Studies

A commercial TrxR (rat liver) solution (4 μM) was diluted with water to yield a 2.86 μM solution. Lyophilized Trx (human) was reconstituted with PBS (10 mM, pH 7.2) yielding a 500 μM solution. A solution of 20 mM NADPH and 10 mM ATG and ATM solutions were prepared in water.

In a black polypropylene 1.5 mL micro centrifuge tube, 437 μL PBS, 25 μL NADPH, 16 μL formulated ocrelizumab solution (30.2 mg/mL) and 5 μL Trx were gently mixed. The reaction was initiated by the addition of 17.5 μL TrxR. The reaction was incubated at room temperature for 24 hours. Aliquots of 20 μL were taken at each sampling time-point and stored at −70° C. until analyzed by the Bioanalyzer assay (see below). Controls were performed to determine if the enzymatic pathway was active when an enzyme was omitted by substituting an equal volume of PBS for either Trx and/or TrxR in the reaction mixture.

Inhibition of the Trx system was demonstrated using the same reaction conditions described above with the addition of 5 μL ATG or ATM. To demonstrate the inhibition of Trx system by Cu^{2+}, 2.5 μL of $CuSO_4$ (10 mM) was added to reaction mixture using the same enzymes but a different buffer (10 mM histidine, 10 mM Na_2SO_4, 137 mM NaCl, 2.5 mM KCl, pH 7.0) to prevent formation of insoluble $Cu_3(PO_4)_2$.

Free Thiol Assay

A standard curve using GSH was generated in PBS (10 mM, pH 6.0±0.05). From a 110 mM GSH solution, standards were prepared at concentrations of 0, 5.5, 11, 22, 44, 55, 110 and 550 μM through serial dilution. From an acetonitrile stock solution of mBB (10 mM stored at −20° C.), a 100 μM solution of mBB was prepared in PBS (10 mM, pH 10.0±0.05) and stored away from light.

In a black, flat bottomed 96 well plate, 100 μL of mBB was dispensed into each well. For the standard curve, 10 μL of standard GSH solution was added yielding a working pH of 8.0±0.2. For samples, 10 μL of sample was added to the wells. All wells were prepared in triplicate. The plate was incubated at room temperature for 1 hour in the dark then read using a fluorescence plate reader (Molecular Devices SpectraMax® Gemini XS) with an excitation wavelength of 390 nm and an emission wavelength of 490 nm. A linear standard curve was generated using the average result of the three standard wells plotted versus GSH concentration. Free thiol levels in samples were calculated from the linear equation of the standard curve using the average value of the three sample wells.

Bioanalyzer Assay

Capillary electrophoresis measurements were acquired using the Agilent 2100 Bioanalyzer. Sample preparation was carried out as described in the Agilent Protein 230 Assay Protocol (manual part number G2938-90052) with minor changes. HCCF samples were diluted, 1:4 and Protein A samples were diluted to 1.0 g/L with water prior to preparation. For HCCF samples at the denaturing step, 24 μL of a 50 mM iodoacetamide (IAM), 0.5% SDS solution was added in addition to the 2 μL of denaturing solution provided. For Protein A samples, 0.5% SDS with no JAM and 2 μL of denaturing solution were used. Digital gel-like images were generated using Agilent 2100 Expert software.

Stock Solutions for HCCF Hold Time Studies

Three separate stock solutions were used in the lab scale HCCF hold time studies: (1) 250 mM stock solution of EDTA (pH 7.4) prepared using EDTA, disodium dihydrate (Mallinckrodt, cat. #7727-06 or Sigma, cat. # E-5134) and EDTA, tetrasodium dihydrate (Sigma, cat. #E-6511), (2) 50 mM stock solution of cupric sulfate pentahydrate ($CuSO_4$, Sigma, cat. # C-8027), and (3) 1 M acetic acid solution (Mallinckrodt, cat. # V193).

Inhibitor Additions and Cell Culture Fluid (CCF) Blending

A stock solution of either 250 mM EDTA or 50 mM $CuSO_4$ was added to the CCF prior to homogenization to evaluate a range of final concentrations to prevent antibody disulfide reduction. Once the final HCCF was generated from the homogenized CCF, these solutions were then mixed with the HCCF generated from the non-homogenized CCF (also containing EDTA or $CuSO_4$) in order to dilute and decrease the total level of cell lysis to below the 100% maximum. Alternatively, a stock solution of 1 M acetic acid was added to a final blended HCCF solution (homogenized CCF and non-homogenized CCF) to decrease the pH of the solution to prevent antibody disulfide reduction.

Approximately 30-50 mL of each HCCF solution (containing EDTA, $CuSO_4$, acetic acid, or no addition for the control) was held in a 50 mL 316L stainless steel vial. The vial was sealed with a clamp, and the solution was not aerated or agitated. The vial was stored at room temperature (18-22° C.). At pre-determined time points, the solution was removed and purified over a lab scale protein A affinity resin.

Similar results can be obtained with other oxidizing agents, such as, for example, cystine and oxidized glutathione.

Air Sparging

To evaluate air sparging of the HCCF generated from homogenized CCF to prevent antibody disulfide reduction, 3-L glass or 15-L stainless steel vessels were utilized. Approximately 1-5 L of HCCF was 0.22 μm sterile filtered into each sterilized vessel. Experimental conditions were maintained at 18-22° C. and 50 (15-L fermentor) or 275 rpm (3-L fermentor) agitation either with or without pH control by the addition of carbon dioxide. Solutions were either sparged with air to increase the dissolved oxygen level to air saturation or with nitrogen (control) to remove any dissolved oxygen in solution. Gas flow to each vessel was variable dependent upon whether a constant aeration rate was used or a minimum level of dissolved oxygen was maintained. At pre-determined time points, 25-50 mL samples were removed from both vessels and purified over a lab scale protein A affinity resin prior to analysis.

Protein A Processing

Antibody in harvested cell culture fluid samples can be captured and purified using a specific affinity chromatography resin. Protein A resin (Millipore, Prosep-vA High Capacity) was selected as the affinity resin for antibody purification. The resin was packed in a 0.66 cm inner diameter glass column (Omnifit®) with a 14 cm bed height resulting in a 4.8 mL final column volume. Chromatography was performed using an AKTA Explorer 100 chromatography system (GE Healthcare).

The resin was exposed to buffers and HCCF at a linear flow rate between 350-560 cm/hr. The resin was equilibrated with 25 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 7.1. For each purification, the resin was loaded between 5-15 mg antibody per mL of resin. The antibody concentration in the HCCF was determined using an immobilized protein A HPLC column (Applied Biosystems, POROS A). After loading, the resin was washed with 25 mM Tris, 25 mM NaCl, 5 mM EDTA, 0.5 M TMAC, pH 7.1, and then the antibody was eluted using 0.1M acetic acid, pH 2.9. Elution pooling was based on UV absorbance at 280 nm measured inline after the column. The purified elution pools were pH-adjusted using 1 M Sodium HEPES to pH 5.0-5.5. After regeneration of the resin with 0.1M phosphoric acid, the same or similar packed resins were used for subsequent purification of other HCCF solutions.

The antibody concentration in the purified protein A pool was measured using UV spectrometry at 280 nm. The purified protein A elution pools were analyzed by the Bioanalyzer assay to quantitate the percentage of intact antibody at 150 kDa molecular weight.

Example 2

Dialysis Experiment

A dialysis experiment was designed and carried out to determine if the reduction of ocrelizumab was caused by small reducing molecules or macromolecules (e.g., enzymes). In this dialysis experiment, purified intact ocrelizumab was placed in a dialysis bag with a molecular weight cut off (MWCO) of 7000 and incubated the dialysis bag in HCCF containing ocrelizumab in a stainless steel mini-tank. As shown in FIGS. **1** and **2**, the ocrelizumab inside the bag was not reduced after the incubation period (FIG. **1**), whereas the ocrelizumab outside the bag in the HCCF was significantly reduced soon after the incubation started. This was evidenced by the loss of intact ocrelizumab (~150 kDa) and the formation of ocrelizumab fragments (various combinations of heavy and light chains) (FIG. **2**). The mass spectrometry analysis of the ocrelizumab in the protein A elution pools from the reduced manufacturing runs indicated that those observed fragments were formed by reduction of only the inter-chain disulfide bonds.

The free thiol measurement showed that no free thiols were present inside the dialysis bag at the beginning of the incubation; however the levels of free thiols inside and outside the dialysis bag become comparable in less than five hours after the incubation started, indicating that the small molecule components in the HCCF are fully equilibrated inside and outside the dialysis bag (FIG. **3**). Since the reduction was observed only outside but not inside the dialysis bag with a MWCO of 7000 Da, the molecular weight of the reducing molecule(s) must be greater than 7000 Da. Thus, an enzymatic reaction is responsible for the reduction of ocrelizumab.

Example 3

Reduction of Ocrelizumab (rhuMAb 2H7, Variant A) by Trx/TrxR In Vitro

The Trx system was tested for its ability to reduce ocrelizumab in vitro by incubating intact ocrelizumab with Trx, TrxR, and NADPH. The Bioanalyzer results indicate that ocrelizumab was reduced in vitro by the Trx system (FIG. **5**). The rate of reduction in this in vitro system appears to be slower than that in the HCCF (for example when compared to the reduction shown in FIG. **2**). This is likely due to lower concentrations of the enzymes (Trx and Trx-R) and/or the buffer system used in the in vitro reaction because reaction rate of Trx system is dependent on both the enzyme concentrations and buffer systems.

Example 4

Inhibitors of the Trx System

(i) Inhibition of Reduction of Recombinant Antibody by Cupric Sulfate

Cupric sulfate is known for its ability to provide oxidizing redox potential and has been used in the cell culture processes to minimize free thiol (i.e., minimize unpaired cysteine) levels in recombinant antibody molecules (Chaderjian et al., 2005, supra). Cupric sulfate was tested for efficacy in inhibiting the Trx system in vitro and the subsequent reduction of ocrelizumab. In this in vitro reduction experiment, the buffer system was changed from PBS to histidine sulfate to avoid the formation of insoluble $Cu_3(PO_4)_2$. FIG. **8** shows that ocrelizumab was readily reduced by the Trx system in the histidine sulfate buffer (even faster than in PBS buffer). The addition of $CuSO_4$ to this reaction clearly inhibits the ocrelizumab reduction (FIG. **9**).

(ii) Inhibition of Reduction of Recombinant Antibody in HCCF by ATG and ATM

Two commercially available specific inhibitors of TrxR, aurothioglucose (ATG) and aurothiomalate (ATM), were tested for their ability to inhibit the Trx system in vitro and the reduction of ocrelizumab. Both ATG and ATM can effectively

inhibit the reduction of ocrelizumab in the assay described above (see FIGS. **6** and **7**). The addition of aurothioglucose or aurothiomalate, at a concentration of 1 mM to the same reaction mixture as described in the caption for FIG. **5** effectively inhibited the ocrelizumab reduction as shown in the digital gel-like image from Bioanalyzer analysis.

If the Trx system was active in the HCCF and reduced ocrelizumab as observed in the manufacturing runs resulting in reduced antibody molecules or in the lab scale experiments, both gold compounds (ATG and ATM) should be able to inhibit the reduction of ocrelizumab in HCCF. FIG. **10** shows that ocrelizumab was readily reduced in an HCCF from homogenized CCT generated from a 3-L fermentor after a period of incubation. However, the ocrelizumab reduction event was completely inhibited when either 1 mM ATG or ATM was added to the HCCF (FIGS. **11** and **12**). These results demonstrated that the Trx system is active in the HCCF and is directly responsible for the reduction of ocrelizumab.

Example 5

The Source of NADPH for Trx System Activity and the Roles of G6P and Glucose in Reduction Mechanism

The reduction of disulfides by the Trx system requires the reducing equivalents from NADPH (FIG. **4**). The main cellular metabolic pathway that provides NADPH for all reductive biosynthesis reactions is the pentose phosphate pathway. For the antibody reduction event to occur, the enzymes in this pathway must be still active in the HCCF in order to keep the Trx system active. At a minimum, the first step in the pentose phosphate pathway (catalyzed by G6PD) must be active to reduce $NADP^+$ to NADPH while converting G6P to 6-phosphogluconolactone. In addition, G6P is most likely produced from glucose and adenosine 5'-triphosphate (ATP) by the hexokinase activity in HCCF. The overall mechanism of ocrelizumab reduction is summarized in FIG. **4**.

The reducing activity in the HCCF appeared to be transitory in some cases and may be inhibited over time under certain storage conditions or after multiple freeze/thaw cycles. HCCF that has fully lost reducing activity provided an opportunity to explore the role of NADPH and G6P in the reduction of ocrelizumab by Trx system.

An HCCF from a large scale manufacturing run (the "beta" run) was subjected to several freeze/thaw cycles and used in an experiment designed to measure reduction; no ocrelizumab reduction was observed (FIG. **13**) despite its ability to bring about antibody reduction seen previously in freshly-thawed HCCF from this same fermentation. NADPH was added to this non-reducing HCCF at a concentration of 5 mM and the reduction event returned (FIG. **14**). Therefore, the Trx system is still intact and active in the HCCF where reduction no longer occurs, and capable of reducing protein and/or antibody if supplied with cofactors. Additionally, the reducing activity was lost over time as the NADPH source was depleted (presumably due to the oxidation of NADPH by all of the reductive reactions that compete for NADPH), and not because the Trx system was degraded or inactivated.

This was verified by another experiment. 10 mM G6P was added to a HCCF that had been repeatedly freeze-thawed from the beta run. This G6P addition reactivated the Trx system which subsequently reduced ocrelizumab in the HCCF incubation experiment (FIG. **15**). This demonstrated that the reduction of ocrelizumab in the HCCF was caused by the activities of both the Trx system and G6PD. Furthermore, G6PD is still active in a repeatedly freeze/thawed HCCF of the beta run; the loss of reduction activity in this a repeatedly freeze/thawed HCCF beta run appears to be due to the depletion of G6P, which thus eliminated the conversion of $NADP^+$ to NADPH.

In our studies, we have observed that EDTA can effectively inhibit the ocrelizumab reduction in the HCCF incubation experiment. As shown in FIG. **16**, the ocrelizumab was reduced after incubating the HCCF from a 12,000 L scale ocrelizumab manufacturing run (not repeatedly freeze/thawed and no loss of reducing activity) at ambient temperature for more than 19 hours. However, the reduction was completely inhibited when 20 mM EDTA was added to the 12 kL HCCF and held in a separate stainless steel minitank (FIG. **17**). In the first step of glycolysis, the hexokinase catalyzes the transfer of phosphate group from Mg2+-ATP to glucose, a reaction that requires the complexation of Mg2+ with ATP (Hammes & Kochavi, 1962a & 1962b, supra). Since EDTA is a metal ion chelator, especially for Mg2+, it can be an effective inhibitor of hexokinase. The observation that an excess amount of EDTA can effectively block the reduction indicates the involvement of hexokinase (i.e. providing G6P) in the mechanism of ocrelizumab reduction. Without being bound by this, or any other theory, EDTA blocks the reduction of ocrelizumab by eliminating the hexokinase activity and thereby reducing the G6P level available for G6PD, and subsequently the NADPH level available for the Trx system.

Although EDTA is every effective in blocking the reduction of ocrelizumab in fresh HCCF, it was unable to prevent the reduction of ocerlizumab in the beta run HCCF in which the Trx system activity was lost then reactivated by the addition of G6P. For example, the reduction of ocrelizumab was observed in an HCCF incubation experiment in which 5 mM G6P and 20 mM EDTA (final concentrations) were added to the beta run HCCF that had fully lost reducing activity (FIG. **18**). However, no reduction was seen in the control incubation experiment in which no G6P and EDTA were added. Without being bound by this or any other theory, the EDTA used in this manner may therefore inhibit neither the Trx system nor the G6PD, and may function as an inhibitor for hexokinase, which produces the G6P for the G6PD. Without G6P, the Trx system would not be supplied with the necessary NADPH for activity.

Example 6

Inhibition of Reduction of Recombinant Antibody by DHEA

Dehydroepiandrosterone (DHEA), as well as other similar G6PD inhibitors, effectively blocks G6PD activity (Gordon et al., 1995, supra). G6PD inhibitors also prevent the reduction of an antibody in HCCF, for example, ocrelizumab, by blocking the generation of NADPH. The ability of DHEA to inhibit the reduction of orcelizumab is demonstrated in an HCCF incubation experiment. Adding DHEA to a HCCF prevents antibody reduction.

DHEA is typically used in the concentration range from about 0.05 mM to about 5 mM. DHEA is also typically used in the concentration range from about 0.1 mM to about 2.5 mM.

Example 7

Inhibition of Reduction of Recombinant Antibody by (i) EDTA, (ii) Cupric Sulfate, and (iii) Acetic Acid Additions

Four different HCCFs were stored and held in the stainless steel vials. The solutions were similar in the amount of cell

lysis, which were generated by diluting HCCF from homogenized CCF with HCCF from non-homogenized CCF. For example, 150 mL of the first lysed solution was mixed with 50 mL of the second solution, respectively. The four HCCF mixtures evaluated in this study contained either: (1) 20 mM EDTA, (2) 30 μM $CuSO_4$, (3) 15 mM acetic acid (pH 5.5), and (4) no chemical inhibitor was added for the control solution. The ocrelizumab antibody from all four mixtures was purified immediately (t=0 hr) using protein A chromatography and then again after 20 hr and 40 hr of storage in the stainless steel vials. Purified protein A elution pools were analyzed by the Bioanalyzer assay to quantitate the percentage of intact antibody (150 kDa). The results showed that greater than 90% intact antibody was present in all four mixtures at the initial time point (FIG. **19**). However, at the 20 hr time point, intact antibody was not detected in the control mixture (without any addition) indicating reduction of the antibody disulfide bonds. In the three other mixtures, over 90% intact antibody was still detected at both 20 hr and 40 hr time points, demonstrating the prevention of disulfide bond reduction by all three inhibitors tested.

Example 8

Inhibition of Reduction of Recombinant Antibody by Air Sparging the HCCF

One HCCF mixture generated from homogenized CCF was stored and held in two separate 10 L stainless steel fermentors. One vessel was sparged with air while the other vessel was sparged with nitrogen gas. The ocrelizumab antibody was purified immediately (t=0 hr) from the initial mixture using protein A chromatography. At selected time points,

50 mL samples were removed from each vessel and the antibody was purified using protein A chromatography. Purified protein A elution pools were then analyzed by the Bioanalyzer assay to quantitate the percentage of intact antibody at 150 kDa. The results showed that approximately 85% intact antibody was present in the initial solution (FIG. **20**), indicating some early reduction of the antibody disulfide bonds prior to exposure to oxygen (i.e. sparged air in the fermentor). Once the mixture was sparged with air for two hours, greater than 90% intact antibody was measured for the remainder of the 36 hr study. In contrast, when the mixture was sparged with nitrogen gas, the antibody reduction event continued as measured at 2 hr (28% 150 kDa peak) and 6 hr (5% 150 kDa peak). These results demonstrated the prevention of disulfide bond reduction in the antibody when the HCCF mixture generated from homogenized CCF was exposed to oxygen.

Example 9

Design of Targeted siRNA or Antisense Nucleotide Trx Inhibitors

The design of targeted siRNAs or antisense nucleotides to the genes as found in CHO cells may be done by using publicly available sequences such as those for *E. coli* thioredoxin TrxA (SEQ ID NO:30), *E. coli* thioredoxin reductase TrxB (SEQ ID NO:31); mouse thioredoxin 1 (SEQ ID NO:32), mouse thioreodoxin 2 (SEQ ID NO:33), mouse thioredoxin reductase 1 (SEQ ID NO:34), and mouse thioredoxin reductase 2 (SEQ ID NO:35). One of ordinary skill in the art can use these sequences to select sequences to design Trx inhibitors for targeting enzymes in different organisms and/or cells, such as CHO cells.

The sequence of *E. coli* Thioredoxin TrxA is:

```
                                                      (SEQ ID NO: 30)
ATG TTA CAC CAA CAA CGA AAC CAA CAC GCC AGG CTT ATT CCT GTG GAG

TTA TAT ATG AGC GAT AAA ATT ATT CAC CTG ACT GAC GAC AGT TTT GAC ACG

GAT GTA CTC AAA GCG GAC GGG GCG ATC CTC GTC GAT TTC TGG GCA GAG

TGG TGC GGT CCG TGC AAA ATG ATC GCC CCG ATT CTG GAT GAA ATC GCT

GAC GAA TAT CAG GGC AAA CTG ACC GTT GCA AAA CTG AAC ATC GAT CAA

AAC CCT GGC ACT GCG CCG AAA TAT GGC ATC CGT GGT ATC CCG ACT CTG CTG

CTG TTC AAA AAC GGT GAA GTG GCG GCA ACC AAA GTG GGT GCA CTG TCT

AAA GGT CAG TTG AAA GAG TTC CTC GAC GCT AAC CTG GCG TAA.
```

The sequence of *E. coli* Thioredoxin TrxB is:

```
                                                      (SEQ ID NO: 31)
ATG GGC ACG ACC AAA CAC AGT AAA CTG CTT ATC CTG GGT TCA GGC CCG

GCG GGA TAC ACC GCT GCT GTC TAC GCG GCG CGC GCC AAC CTG CAA CCT

GTG CTG ATT ACC GGC ATG GAA AAA GGC GGC CAA CTG ACC ACC ACC ACG

GAA GTG GAA AAC TGG CCT GGC GAT CCA AAC GAT CTG ACC GGT CCG TTA

TTA ATG GAG CGC ATG CAC GAA CAT GCC ACC AAG TTT GAA ACT GAG ATC

ATT TTT GAT CAT ATC AAC AAG GTG GAT CTG CAA AAC CGT CCG TTC CGT CTG

AAT GGC GAT AAC GGC GAA TAC ACT TGC GAC GCG CTG ATT ATT GCC ACC

GGA GCT TCT GCA CGC TAT CTC GGC CTG CCC TCT GAA GAA GCC TTT AAA GGC
```

-continued

CGT GGG GTT TCT GCT TGT GCA ACC TGC GAC GGT TTC TTC TAT CGC AAC CAG

AAA GTT GCG GTC ATC GGC GGC GGC AAT ACC GCG GTT GAA GAG GCG TTG

TAT CTG TCT AAC ATC GCT TCG GAA GTG CAT CTG ATT CAC CGC CGT GAC GGT

TTC CGC GCG GAA AAA ATC CTC ATT AAG CGC CTG ATG GAT AAA GTG GAG

AAC GGC AAC ATC ATT CTG CAC ACC AAC CGT ACG CTG GAA GAA GTG ACC

GGC GAT CAA ATG GGT GTC ACT GGC GTT CGT CTG CGC GAT ACG CAA AAC

AGC GAT AAC ATC GAG TCA CTC GAC GTT GCC GGT CTG TTT GTT GCT ATC GGT

CAC AGC CCG AAT ACT GCG ATT TTC GAA GGG CAG CTG GAA CTG GAA AAC

GGC TAC ATC AAA GTA CAG TCG GGT ATT CAT GGT AAT GCC ACC CAG ACC

AGC ATT CCT GGC GTC TTT GCC GCA GGC GAC GTG ATG GAT CAC ATT TAT CGC

CAG GCC ATT ACT TCG GCC GGT ACA GGC TGC ATG GCA GCA CTT GAT GCG

GAA CGC TAC CTC GAT GGT TTA GCT GAC GCA AAA TAA.

The sequence of mouse thioredoxin 1 is:

(SEQ ID NO: 32)

ATGGTGAAGCTGATCGAGAGCAAGGAAGCTTTTCAGGAGGCCCTGGCCGC

CGCGGGAGACAAGCTTGTCGTGGTGGACTTCTCTGCTACGTGGTGTGGAC

CTTGCAAAATGATCAAGCCCTTCTTCCATTCCCTCTGTGACAAGTATTCC

AATGTGGTGTTCCTTGAAGTGGATGTGGATGACTGCCAGGATGTTGCTGC

AGACTGTGAAGTCAAATGCATGCCGACCTTCCAGTTTTATAAAAAGGGTC

AAAAGGTGGGGGAGTTCTCCGGTGCTAACAAGGAAAAGCTTGAAGCCTCT

ATTACTGAATATGCCTAA.

The sequence of mouse thioreodoxin 2 is:

(SEQ ID NO: 33)

ATGGCTCAGCGGCTCCTCCTGGGGAGGTTCCTGACCTCAGTCATCTCCAG

GAAGCCTCCTCAGGGTGTGTGGGCTTCCCTCACCTCTAAGACCCTGCAGA

CCCCTCAGTACAATGCTGGTGGTCTAACAGTAATGCCCAGCCCAGCCCGG

ACAGTACACACCACCAGAGTCTGTTTGACGACCTTTAACGTCCAGGATGG

ACCTGACTTTCAAGACAGAGTTGTCAACAGTGAGACACCAGTTGTTGTGG

ACTTTCATGCACAGTGGTGTGGCCCCTGCAAGATCCTAGGACCGCGGCTA

GAGAAGATGGTCGCCAAGCAGCACGGGAAGGTGGTCATGGCCAAAGTGGA

CATTGACGATCACACAGACCTTGCCATTGAATATGAGGTGTCAGCTGTGC

CTACCGTGCTAGCCATCAAGAACGGGGACGTGGTGGACAAGTTTGTGGGG

ATCAAGGACGAGGACCAGCTAGAAGCCTTCCTGAAGAAGCTGATTGGCTG

A.

The sequence of mouse thioredoxin reductase 1 is:

(SEQ ID NO: 34)

ATGAATGGCTCCAAAGATCCCCCTGGGTCCTATGACTTCGACCTGATCAT

CATTGGAGGAGGCTCAGGAGGACTGGCAGCAGCTAAGGAGGCAGCCAAAT

TTGACAAGAAAGTGCTGGTCTTGGATTTTGTCACACCGACTCCTCTTGGG

-continued

ACCAGATGGGGTCTCGGAGGAACGTGTGTGAATGTGGGTTGCATACCTAA

GAAGCTGATGCACCAGGCAGCTTTGCTCGGACAAGCTCTGAAAGACTCGC

GCAACTATGGCTGGAAAGTCGAAGACACAGTGAAGCATGACTGGGAGAAA

ATGACGGAATCTGTGCAGAGTCACATCGGCTCGCTGAACTGGGGCTACCG

CGTAGCTCTCCGGGAGAAAAAGGTCGTCTATGAGAATGCTTACGGGAGGT

TCATTGGTCCTCACAGGATTGTGGCGACAAATAACAAAGGTAAAGAAAAA

ATCTATTCAGCAGAGCGGTTCCTCATCGCCACAGGTGAGAGGCCCCGCTA

CCTGGGCATCCCTGGAGACAAAGAGTACTGCATCAGCAGTGATGATCTTT

TCTCCTTGCCTTACTGCCCGGGGAAGACCCTAGTAGTTGGTGCATCCTAT

GTCGCCTTGGAATGTGCAGGATTTCTGGCTGGTATCGGCTTAGACGTCAC

TGTAATGGTGCGGTCCATTCTCCTTAGAGGATTTGACCAAGACATGGCCA

ACAAAATCGGTGAACACATGGAAGAACATGGTATCAAGTTTATAAGGCAG

TTCGTCCCAACGAAAATTGAACAGATCGAAGCAGGAACACCAGGCCGACT

CAGGGTGACTGCTCAATCCACAAACAGCGAGGAGACCATAGAGGGCGAAT

TTAACACAGTGTTGCTGGCGGTAGGAAGAGATTCTTGTACGAGAACTATT

GGCTTAGAGACCGTGGGCGTGAAGATAAACGAAAAAACCGGAAAGATACC

CGTCACGGATGAAGAGCAGACCAATGTGCCTTACATCTACGCCATCGGTG

ACATCCTGGAGGGGAAGCTAGAGCTGACTCCCGTAGCCATCCAGGCGGGG

AGATTGCTGGCTCAGAGGCTGTATGGAGGCTCCAATGTCAAATGTGACTA

TGACAATGTCCCAACGACTGTATTTACTCCTTTGGAATATGGCTGTTGTG

GCCTCTCTGAAGAAAAAGCCGTAGAGAAATTTGGGGAAGAAAATATTGAA

GTTTACCATAGTTTCTTTTGGCCATTGGAATGGACAGTCCCATCCCGGGA

TAACAACAAATGTTATGCAAAAATAATCTGCAACCTTAAAGACGATGAAC

GTGTCGTGGGCTTCCACGTGCTGGGTCCAAACGCTGGAGAGGTGACGCAG

GGCTTTGCGGCTGCGCTCAAGTGTGGGCTGACTAAGCAGCAGCTGGACAG

CACCATCGGCATCCACCCGGTCTGTGCAGAGATATTCACAACGTTGTCAG

TGACGAAGCGCTCTGGGGGAGACATCCTCCAGTCTGGCTGCTGA

The sequence of mouse thioredoxin reductase 2 is:

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                                         (SEQ ID NO: 35)
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GCGCTTCCGGCCGCGGACACGGGCTCTGACACGCGGGACAAGGGGCGCGG

CGAGTGCAGCGGGAGGGCAGCAGAGCTTTGATCTCTTGGTGATCGGTGGG

GGATCCGGTGGCCTAGCTTGTGCCAAGGAAGCTGCTCAGCTGGGAAAGAA

GGTGGCTGTGGCTGACTATGTGGAACCTTCTCCCCGAGGCACCAAGTGGG

GCCTTGGTGGCACCTGTGTCAACGTGGGTTGCATACCCAAGAAGCTGATG

CATCAGGCTGCACTGCTGGGGGGCATGATCAGAGATGCTCACCACTATGG

CTGGGAGGTGGCCCAGCCTGTCCAACACAACTGGAAGACAATGGCAGAAG

CCGTGCAAAACCATGTGAAATCCTTGAACTGGGGTCATCGCGTCCAACTG

CAGGACAGGAAAGTCAAGTACTTTAACATCAAAGCCAGCTTTGTGGATGA

GCACACAGTTCGCGGTGTGGACAAAGGCGGGAAGGCGACTCTGCTTTCAG

CTGAGCACATTGTCATTGCTACAGGAGGACGGCCAAGGTACCCCACACAA

GTCAAAGGAGCCCTGGAATATGGAATCACAAGTGACGACATCTTCTGGCT

GAAGGAGTCCCCTGGGAAAACGTTGGTGGTTGGAGCCAGCTATGTGGCCC

TAGAGTGTGCTGGCTTCCTCACTGGAATTGGACTGGATACCACTGTCATG

ATGCGCAGCATCCCTCTCCGAGGCTTTGACCAGCAAATGTCATCTTTGGT

CACAGAGCACATGGAGTCTCATGGCACCCAGTTCCTGAAAGGCTGTGTCC

CCTCCCACATCAAAAAACTCCCAACTAACCAGCTGCAGGTCACTTGGGAG

GATCATGCTTCTGGCAAGGAAGACACAGGCACCTTTGACACTGTCCTGTG

GGCCATAGGGCGAGTTCCAGAAACCAGGACTTTGAATCTGGAGAAGGCTG

GCATCAGTACCAACCCTAAGAATCAGAAGATTATTGTGGATGCCCAGGAG

GCTACCTCTGTTCCCCACATCTATGCCATTGGAGATGTTGCTGAGGGGCG

GCCTGAGCTGACGCCCACAGCTATCAAGGCAGGAAAGCTTCTGGCTCAGC

GGCTCTTTGGGAAATCCTCAACCTTAATGGATTACAGCAATGTTCCCACA

ACTGTCTTTACACCACTGGAGTATGGCTGTGTGGGGCTGTCTGAGGAGGA

GGCTGTGGCTCTCCATGGCCAGGAGCATGTAGAGGTTTACCATGCATATT

ATAAGCCCCTAGAGTTCACGGTGGCGGATAGGGATGCATCACAGTGCTAC

ATAAAGATGGTATGCATGAGGGAGCCCCCACAACTGGTGCTGGGCCTGCA

CTTCCTTGGCCCCAACGCTGGAGAAGTCACCCAAGGATTTGCTCTTGGGA

TCAAGTGTGGGGCTTCATATGCACAGGTGATGCAGACAGTAGGGATCCAT

CCCACCTGCTCTGAGGAGGTGGTCAAGCTGCACATCTCCAAGCGCTCCGG

CCTGGAGCCTACTGTGACTGGTTGCTGA.
```

Example 10

In Vitro Trx/Trx Reductase Studies

Materials and Methods

A commercial TrxR (rat liver) solution (4 μM) was diluted with water to yield a 2.86 μM solution. Lyophilized Trx (human) was reconstituted with PBS (10 mM, pH 7.2) yielding a 500 μM solution. A solution of 20 mM NADPH and 10 mM ATG and ATM solutions were prepared in water.

In a black polypropylene 1.5 mL micro centrifuge tube, 437 μL reaction buffer (10 mM histidine, 10 mM Na2SO4, 137 mM NaCl, 2.5 mM KCl, pH 7.0), 25 μL NADPH, 16 μL formulated ocrelizumab solution (30.2 mg/mL) and 5 μL Trx were gently mixed. The reaction was initiated by the addition of 17.5 μL TrxR. The reaction was incubated at room temperature for 24 hours. Aliquots of 20 μL were taken at each sampling time-point and stored at −70° C. until analyzed by the Bioanalyzer assay.

Inhibition of the Trx system was demonstrated using the same reaction conditions described above with the addition of various inhibitors.

1. In Vitro Activity of Thioredoxin System

FIG. **24** shows a digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that incubation of intact ocrelizumab ("2H7," a humanized anti-CD20 antibody, referred to as "Variant A" above) (1 mg/mL) with 0.1 μM TrxR (rat liver), 5 μM Trx (human) and 1 mM NADPH in 10 mM histidine sulfate buffer results in the reduction of ocrelizumab in less than one hour.

2. In Vitro Activity of Thioredoxin System Inhibited by Aurothioglucose

Aurothioglucose (ATG) was added to the ocrelizumab mixture described above, at the following concentrations: 1 mM; 0.6 μM (6:1 ATG:TrxR); 0.4 μM (4:1 ATG:TrxR); and 0.2 μM (2:1 ATG:TrxR).

As attested by the digital gel-like images from Bioanalyzer analysis shown in FIGS. **25**-**27**, aurothioglucose added at concentrations 1 mM, 0.6 μM, and 0.4 μM effectively inhibits the reduction of ocrelizumab by the thioredoxin system. However, as shown in FIG. **28**, under these experimental conditions aurothioglucose added at a concentration of 0.2 μM cannot inhibit ocrelizumab reduction after 24 hours.

3. In Vitro Activity of Thioredoxin System Inhibited by Aurothiomalate

Aurothiomalate (ATM) was added to the ocrelizumab mixture described above, at concentrations of 0.1 mM and 0.01 mM. As attested by the digital gel-like images from Bioanalyzer analysis shown in FIGS. **29** and **30**, ATM effectively inhibits the reduction of ocrelizumab by the thioredoxin system at both concentrations tested.

4. In Vitro Activity of Thioredoxin System Inhibited by $Cuso_4$

$CuSO_4$ was added to the ocrelizumab mixture described above, at concentrations of 20 μM (4:1 Cu^{2+}:Trx); 10 μm (2:1 Cu^{2+}:Trx); and 5 μM (1:1 Cu^{2+}:Trx). As shown in FIGS. **31**-**33**, $CuSO_4$ effectively inhibits thioredoxin-induced reduction of ocrelizumab at concentrations of 20 μM and 10 μM (FIGS. **31** and **32**), but the 5 μM concentration is insufficient to result in a complete inhibition of reduction (FIG. **33**).

5. In Vitro Activity of Thioredoxin System Inhibited by Cystamine

Cystamine was added to the ocrelizumab mixture describe above at the following concentrations: 532 μM (20:1 cystamine:2H7 (Variant A) disulfide); 266 μM (10:1 cystamine:2H7 (Variant A) disulfide); 133 μM (5:1 cystamine:2H7 disulfide); and 26.6 μM (1:1 cystamine:2H7 (Variant A) disulfide). As shown in FIGS. **34**-**37**, cystamine effectively inhibits thioredoxin-induced reduction of ocrelizumab at concentrations of 532 μM (20:1 cystamine:2H7 (Variant A) disulfide) and 266 μM (10:1 cystamine:2H7 (Variant A)) (FIGS. **34** and **35**) but the 133 μM (5:1 cystamine:2H7 (Variant A) disulfide) and 26.6 μM (1:1 cystamine:2H7 (Variant A) disulfide) concentrations are insufficient to inhibit the reduction of ocrelizumab after 24 hours (FIGS. **36** and **37**).

6. In Vitro Activity of Thioredoxin System Inhibited by Cystine

Cystine was added to the ocrelizumab mixture described above at a concentration of 2.6 mM. As shown in FIG. **38**, at this concentration cystine effectively inhibits reduction of ocrelizumab by the thioredoxin system. It is noted that the minimum effective concentration of cystine (just as the effective minimum concentration of other inhibitors) depends on the actual circumstances, and might be different for different proteins, such as antibodies, and might vary depending on the timing of addition. Thus, for example, if cystine is added pre-lysis, the minimum effective concentration for antibody 2H7 (Variant A) is about 1.3 mM, for Apomab about 1 mM and for antibody Variant C about 4.5 mM. When cystine is added in the cell culture medium, the minimum effective concentration typically is somewhat higher, and is about 5.2 mM for 2H7 (Variant A), 6 mM for Apomab and 9 mM for antibody Variant C. Usually, for cystine, cystamine and oxidized glutathione (see below) the minimum effective inhibitory concentration is about 40× of the antibody concentration (in μM).

7. In Vitro Activity of Thioredoxin System Inhibited by Oxidized Glutathione (GSSG)

GSSG was added to the ocrelizumab mixture described above at a concentration of 2.6 mM. As shown in FIG. **39**, at this concentration GSSG effectively inhibits reduction of ocrelizumab by the thioredoxin system. It is noted, however, that the minimum effective concentration of oxidize glutathione (just as that of the other inhibitors) depends on the actual circumstances, such as, for example, on the nature of the protein (e.g. antibody) produced and the timing of addition. For example, for antibody 2H7 (Variant A) the minimum effective concentration is about 1.3 mM for addition prior to lysis.

8. In Vitro Activity of Enzymatic Reduction System

FIG. **40** shows a digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that incubation of intact ocrelizumab ("2H7," a humanized anti-CD20 antibody, Variant A) (1 mg/mL) with 10 μg/mL hexokinase, 50 μg/mL glucose-6-phosphate dehydrogenase, 5 μM thioredoxin, 0.1 μM thoredoxin reductase, 2 mM glucose, 0.6 mM ATP, 2 mM Mg^{2+}, and 2 mM NADP in 50 mM histidine sulfate buffered at pH 7.38 results in the reduction of ocrelizumab in about one hour. Addition of 0.1 mM HDEA, a known glucose-6-phosphate dehydrogenase inhibitor does not inhibit the reduction.

9. In Vitro Activity of Enzymatic Reduction System Requires NADPH

As shown in the digital gel-like image from Bioanalyzer analysis of FIG. **41**, incubation of intact ocrelizumab (1 mg/mL) with 5 μM thioredoxin, 0.1 μM thioredoxin reductase, and 2 mM NADP in 50 mM histidine sulfate buffer at pH 7.38 does not result in the reduction of the ocrelizumab antibody. Reduction of ocrelizumab could not occur without hexokinase and glucose-6-phosphate dehydrogenase and their substrates to generate NADPH.

The invention illustratively described herein can suitably be practiced in the absence of any element or elements, limitation or limitations that is not specifically disclosed herein. Thus, for example, the terms "comprising," "including," "containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalent of the invention shown or portion thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modifications and variations of the inventions embodied herein disclosed can be readily made by those skilled in the art, and that such modifications and variations are considered to be within the scope of the inventions disclosed herein. The inventions have been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form the part of these inventions. This includes within the generic description of each of the inventions a proviso or negative limitation that will allow removing any subject matter from the genus, regardless or whether or not the material to be removed was specifically recited. In addition, where features or aspects of an invention are described in terms of the Markush group, those schooled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. Further, when a reference to an aspect of the invention lists a range of individual members, as for example, 'SEQ ID NO:1 to SEQ ID NO:100, inclusive,' it is intended to be equivalent to listing every member of the list individually, and additionally it should be understood that every individual member may be excluded or included in the claim individually.

The steps depicted and/or used in methods herein may be performed in a different order than as depicted and/or stated. The steps are merely exemplary of the order these steps may occur. The steps may occur in any order that is desired such that it still performs the goals of the claimed invention.

From the description of the invention herein, it is manifest that various equivalents can be used to implement the concepts of the present invention without departing from its scope. Moreover, while the invention has been described with specific reference to certain embodiments, a person of ordinary skill in the art would recognize that changes can be made in form and detail without departing from the spirit and the scope of the invention. The described embodiments are considered in all respects as illustrative and not restrictive. It should also be understood that the invention is not limited to the particular embodiments described herein, but is capable of many equivalents, rearrangements, modifications, and substitutions without departing from the scope of the invention. Thus, additional embodiments are within the scope of the invention and within the following claims.

All U.S. patents and applications; foreign patents and applications; scientific articles; books; and publications mentioned herein are hereby incorporated by reference in their entirety as if each individual patent or publication was specifically and individually indicated to be incorporated by reference, including any drawings, figures and tables, as though set forth in full.

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Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
        35                  40                  45

Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Phe Asn Pro Pro Thr
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Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
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<220> FEATURE:
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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr Phe Asp Val Trp
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Gly Gln Gly Thr Leu Val Thr Val Ser Ser
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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Leu
            20                  25                  30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
        35                  40                  45
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-continued

Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
50 55 60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
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Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ala Phe Asn Pro Pro Thr
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Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
100 105

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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain variable

<400> SEQUENCE: 4

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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
50 55 60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
100 105 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

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<211> LENGTH: 122
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain variable

<400> SEQUENCE: 5

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1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
50 55 60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Val Val Tyr Tyr Ser Tyr Arg Tyr Trp Tyr Phe Asp Val Trp
100 105 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

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<211> LENGTH: 213
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1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Met
            20                  25                  30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
        35                  40                  45

Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Phe Asn Pro Pro Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
            100                 105                 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
        115                 120                 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
    130                 135                 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145                 150                 155                 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
                165                 170                 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
            180                 185                 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
        195                 200                 205

Asn Arg Gly Glu Cys
    210


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<220> FEATURE:
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<400> SEQUENCE: 7

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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95
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Ala Arg Val Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335

Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 8
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1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430
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Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
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<220> FEATURE:
<223> OTHER INFORMATION: full length light chain

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Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Leu
            20                  25                  30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
        35                  40                  45

Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ala Phe Asn Pro Pro Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
            100                 105                 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
        115                 120                 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
    130                 135                 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145                 150                 155                 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
                165                 170                 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
            180                 185                 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
        195                 200                 205

Asn Arg Gly Glu Cys
    210


<210> SEQ ID NO 10
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain

<400> SEQUENCE: 10

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60
```

```
Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450
```

<210> SEQ ID NO 11
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial

```
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain sequence

<400> SEQUENCE: 11

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Ala Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335

Ile Glu Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400
```

```
Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 12
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain sequence

<400> SEQUENCE: 12

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
```

305 310 315 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
325 330 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
340 345 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
355 360 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
370 375 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385 390 395 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
405 410 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
420 425 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
435 440 445

Ser Pro Gly Lys
450

<210> SEQ ID NO 13
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain sequence

<400> SEQUENCE: 13

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
50 55 60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
100 105 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
115 120 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
130 135 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145 150 155 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
165 170 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
180 185 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
195 200 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
210 215 220

```
Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Trp His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 14
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain

<400> SEQUENCE: 14

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Tyr Arg Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125
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```
Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 15
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain

<400> SEQUENCE: 15

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
```

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```
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450
```

<210> SEQ ID NO 16
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 16

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Ser Ile Gly
            20                  25                  30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Tyr Ile Tyr Pro Tyr
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105
```

<210> SEQ ID NO 17
<211> LENGTH: 119
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 17

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr Asp Tyr
            20                  25                  30

Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr Asn Gln Arg Phe
    50                  55                  60

Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Asn Leu Gly Pro Ser Phe Tyr Phe Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Leu Val Thr Val Ser Ser
        115
```

<210> SEQ ID NO 18
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: light chain

<400> SEQUENCE: 18

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn Thr Ala
            20                  25                  30
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```
Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Tyr Thr Thr Pro Pro
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105


<210> SEQ ID NO 19
<211> LENGTH: 120
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain

<400> SEQUENCE: 19

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys Asp Thr
            20                  25                  30

Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys Asn Thr Ala Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ser Arg Trp Gly Gly Asp Gly Phe Tyr Ala Met Asp Tyr Trp Gly Gln
            100                 105                 110

Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 20
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 20

Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu Gly
1               5                   10                  15

Asp Arg Val Ile Ile Ser Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
            20                  25                  30

Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys Val Leu Ile
        35                  40                  45

Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu Pro
65                  70                  75                  80

Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
                85                  90                  95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
            100                 105
```

```
<210> SEQ ID NO 21
<211> LENGTH: 123
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 21

Glu Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Gln Pro Gly Glu
1               5                   10                  15

Thr Val Arg Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
            20                  25                  30

Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu Lys Trp Met
        35                  40                  45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
    50                  55                  60

Lys Arg Arg Phe Thr Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Tyr
65                  70                  75                  80

Leu Gln Ile Ser Asn Leu Lys Asn Asp Asp Thr Ala Thr Tyr Phe Cys
                85                  90                  95

Ala Lys Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
            100                 105                 110

Trp Gly Ala Gly Thr Thr Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 22
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 22

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
            20                  25                  30

Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Val Leu Ile
        35                  40                  45

Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
            100                 105


<210> SEQ ID NO 23
<211> LENGTH: 123
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 23

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
```

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```
            20                  25                  30

Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
    50                  55                  60

Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Lys Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
            100                 105                 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 24
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 24

Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
            20                  25                  30

Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Val Leu Ile
        35                  40                  45

Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
            100                 105


<210> SEQ ID NO 25
<211> LENGTH: 123
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 25

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
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Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Asp Phe Thr His Tyr
            20                  25                  30

Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
    50                  55                  60

Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Lys Tyr Pro Tyr Tyr Tyr Gly Thr Ser His Trp Tyr Phe Asp Val
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            100                 105                 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
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<210> SEQ ID NO 26
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 26

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
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Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Lys Thr Ile Ser Lys Tyr
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Ser Gly Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Asn Glu Tyr Pro Leu
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
            100                 105


<210> SEQ ID NO 27
<211> LENGTH: 121
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 27

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr Gly His
            20                  25                  30

Trp Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Gly Ile Tyr Phe Tyr Gly Thr Thr Tyr Phe Asp Tyr Trp Gly
            100                 105                 110

Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 28
<211> LENGTH: 214
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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 28
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Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Lys Thr Ile Ser Lys Tyr
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Ser Gly Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Asn Glu Tyr Pro Leu
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
            100                 105                 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
        115                 120                 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
    130                 135                 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145                 150                 155                 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
                165                 170                 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
            180                 185                 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
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Phe Asn Arg Gly Glu Cys
    210


<210> SEQ ID NO 29
<211> LENGTH: 451
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 29

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr Gly His
            20                  25                  30

Trp Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Gly Ile Tyr Phe Tyr Gly Thr Thr Tyr Phe Asp Tyr Trp Gly
            100                 105                 110

Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser
        115                 120                 125

Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala
    130                 135                 140

Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
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145                 150                 155                 160

Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala
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Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
            180                 185                 190

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His
        195                 200                 205

Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys
    210                 215                 220

Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly
225                 230                 235                 240

Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
                245                 250                 255

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
            260                 265                 270

Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val
        275                 280                 285

His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr
    290                 295                 300

Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly
305                 310                 315                 320

Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile
                325                 330                 335

Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val
            340                 345                 350

Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser
        355                 360                 365

Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
    370                 375                 380

Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
385                 390                 395                 400

Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val
                405                 410                 415

Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
            420                 425                 430

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser
        435                 440                 445

Pro Gly Lys
    450

<210> SEQ ID NO 30
<211> LENGTH: 384
<212> TYPE: DNA
<213> ORGANISM: E. coli Thioredoxin TrxA

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gataaaatta ttcacctgac tgacgacagt tttgacacgg atgtactcaa agcggacggg     120

gcgatcctcg tcgatttctg ggcagagtgg tgcggtccgt gcaaaatgat cgccccgatt     180

ctggatgaaa tcgctgacga atatcagggc aaactgaccg ttgcaaaact gaacatcgat     240

caaaaccctg gcactgcgcc gaaatatggc atccgtggta tcccgactct gctgctgttc     300

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ttcctcgacg ctaacctggc gtaa                                            384
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<210> SEQ ID NO 31
<211> LENGTH: 966
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ggcggccaac tgaccaccac cacggaagtg gaaaactggc ctggcgatcc aaacgatctg 180

accggtccgt tattaatgga gcgcatgcac gaacatgcca ccaagtttga aactgagatc 240

atttttgatc atatcaacaa ggtggatctg caaaaccgtc cgttccgtct gaatggcgat 300

aacggcgaat acacttgcga cgcgctgatt attgccaccg gagcttctgc acgctatctc 360

ggcctgccct ctgaagaagc ctttaaaggc cgtggggttt ctgcttgtgc aacctgcgac 420

ggtttcttct atcgcaacca gaaagttgcg gtcatcggcg gcggcaatac cgcggttgaa 480

gaggcgttgt atctgtctaa catcgcttcg gaagtgcatc tgattcaccg ccgtgacggt 540

ttccgcgcgg aaaaaatcct cattaagcgc ctgatggata aagtggagaa cggcaacatc 600

attctgcaca ccaaccgtac gctggaagaa gtgaccggcg atcaaatggg tgtcactggc 660

gttcgtctgc gcgatacgca aaacagcgat aacatcgagt cactcgacgt tgccggtctg 720

tttgttgcta tcggtcacag cccgaatact gcgattttcg aagggcagct ggaactggaa 780

aacggctaca tcaaagtaca gtcgggtatt catggtaatg ccacccagac cagcattcct 840

ggcgtctttg ccgcaggcga cgtgatggat cacatttatc gccaggccat tacttcggcc 900

ggtacaggct gcatggcagc acttgatgcg gaacgctacc tcgatggttt agctgacgca 960

aaataa 966

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<211> LENGTH: 318
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aagcttgtcg tggtggactt ctctgctacg tggtgtggac cttgcaaaat gatcaagccc 120

ttcttccatt ccctctgtga caagtattcc aatgtggtgt tccttgaagt ggatgtggat 180

gactgccagg atgttgctgc agactgtgaa gtcaaatgca tgccgacctt ccagttttat 240

aaaaagggtc aaaaggtggg ggagttctcc ggtgctaaca aggaaaagct tgaagcctct 300

attactgaat atgcctaa 318

<210> SEQ ID NO 33
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<212> TYPE: DNA
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<400> SEQUENCE: 33

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cagggtgtgt gggcttccct cacctctaag accctgcaga cccctcagta caatgctggt 120

ggtctaacag taatgcccag cccagcccgg acagtacaca ccaccagagt ctgtttgacg 180

acctttaacg tccaggatgg acctgacttt caagacagag ttgtcaacag tgagacacca 240

gttgttgtgg actttcatgc acagtggtgt ggcccctgca agatcctagg accgcggcta 300

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gagaagatgg tcgccaagca gcacgggaag gtggtcatgg ccaaagtgga cattgacgat    360

cacacagacc ttgccattga atatgaggtg tcagctgtgc ctaccgtgct agccatcaag    420

aacggggacg tggtggacaa gtttgtgggg atcaaggacg aggaccagct agaagccttc    480

ctgaagaagc tgattggctg a                                              501


<210> SEQ ID NO 34
<211> LENGTH: 1494
<212> TYPE: DNA
<213> ORGANISM: mus musculus

<400> SEQUENCE: 34

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ggctcaggag gactggcagc agctaaggag gcagccaaat ttgacaagaa agtgctggtc    120

ttggattttg tcacaccgac tcctcttggg accagatggg gtctcggagg aacgtgtgtg    180

aatgtgggtt gcatacctaa gaagctgatg caccaggcag ctttgctcgg acaagctctg    240

aaagactcgc gcaactatgg ctggaaagtc gaagacacag tgaagcatga ctgggagaaa    300

atgacggaat ctgtgcagag tcacatcggc tcgctgaact ggggctaccg cgtagctctc    360

cgggagaaaa aggtcgtcta tgagaatgct tacgggaggt tcattggtcc tcacaggatt    420

gtggcgacaa ataacaaagg taaagaaaaa atctattcag cagagcggtt cctcatcgcc    480

acaggtgaga ggccccgcta cctgggcatc cctggagaca aagagtactg catcagcagt    540

gatgatcttt tctccttgcc ttactgcccg gggaagaccc tagtagttgg tgcatcctat    600

gtcgccttgg aatgtgcagg atttctggct ggtatcggct tagacgtcac tgtaatggtg    660

cggtccattc tccttagagg atttgaccaa gacatggcca acaaaatcgg tgaacacatg    720

gaagaacatg gtatcaagtt tataaggcag ttcgtcccaa cgaaaattga acagatcgaa    780

gcaggaacac caggccgact cagggtgact gctcaatcca caaacagcga ggagaccata    840

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gagctgactc ccgtagccat ccaggcgggg agattgctgg ctcagaggct gtatggaggc   1080

tccaatgtca aatgtgacta tgacaatgtc ccaacgactg tatttactcc tttggaatat   1140

ggctgttgtg gcctctctga agaaaaagcc gtagagaaat ttggggaaga aaatattgaa   1200

gtttaccata gtttcttttg gccattggaa tggacagtcc catcccggga taacaacaaa   1260

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actaagcagc agctggacag caccatcggc atccacccgg tctgtgcaga gatattcaca   1440

acgttgtcag tgacgaagcg ctctgggggga gacatcctcc agtctggctg ctga        1494


<210> SEQ ID NO 35
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<213> ORGANISM: mus musculus

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cagagctttg atctcttggt gatcggtggg ggatccggtg gcctagcttg tgccaaggaa    180
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gctgctcagc tgggaaagaa ggtggctgtg gctgactatg tggaaccttc tccccgaggc    240

accaagtggg gccttggtgg cacctgtgtc aacgtgggtt gcatacccaa gaagctgatg    300

catcaggctg cactgctggg gggcatgatc agagatgctc accactatgg ctgggaggtg    360

gcccagcctg tccaacacaa ctggaagaca atggcagaag ccgtgcaaaa ccatgtgaaa    420

tccttgaact ggggtcatcg cgtccaactg caggacagga aagtcaagta ctttaacatc    480

aaagccagct ttgtggatga gcacacagtt cgcggtgtgg acaaaggcgg gaaggcgact    540

ctgctttcag ctgagcacat tgtcattgct acaggaggac ggccaaggta ccccacacaa    600

gtcaaaggag ccctggaata tggaatcaca agtgacgaca tcttctggct gaaggagtcc    660

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cagcaaatgt catctttggt cacagagcac atggagtctc atggcaccca gttcctgaaa    840

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cccacctgct ctgaggaggt ggtcaagctg cacatctcca agcgctccgg cctggagcct   1560

actgtgactg gttgctga                                                 1578
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What is claimed is:

1. A method for the prevention of the reduction of a disulfide bond in an antibody expressed in a recombinant host cell, comprising, following fermentation, sparging the pre-harvest or harvested culture fluid of said recombinant host cell with air, wherein the amount of dissolved oxygen (dO_2) in the pre-harvest or harvested culture fluid is at least 10%.

2. The method of claim **1** wherein said air sparging is continued until the pre-harvest or harvested culture fluid is at least 30% saturated with air.

3. The method of claim **1** wherein said air sparging is continued until the pre-harvest or harvested culture fluid is between about 100% saturated to about 30% saturated with air.

4. The method of claim **1** wherein the amount of dissolved oxygen (dO_2) in the pre-harvest or harvested culture fluid is at least 30%.

5. The method of claim **1** wherein the antibody is a therapeutic antibody.

6. The method of claim **1** wherein the antibody is a biologically functional fragment of an antibody.

7. The method of claim **1** wherein the host cell is eukaryotic host cell.

8. The method of claim **7** wherein the eukaryotic host cell is a mammalian host cell.

9. The method of claim **1** wherein the host cell is prokaryotic host cell.

10. The method of claim **9** wherein the prokaryotic host cell is a bacterial cell.

* * * * *

EXHIBIT DD

US008771988B2

(12) **United States Patent**
Goepfert et al.

(10) **Patent No.: US 8,771,988 B2**
(45) **Date of Patent: Jul. 8, 2014**

(54) **PROTEIN EXPRESSION FROM MULTIPLE NUCLEIC ACIDS**

(75) Inventors: **Ulrich Goepfert**, Munich (DE); **Hendrik Knoetgen**, Penzberg (DE); **Erhard Kopetzki**, Penzberg (DE); **Anne Stern**, Penzberg (DE)

(73) Assignee: **Hoffmann-La Roche Inc.**, Nutley, NJ (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 407 days.

(21) Appl. No.: **12/681,781**

(22) PCT Filed: **Oct. 9, 2008**

(86) PCT No.: **PCT/EP2008/008523**

§ 371 (c)(1), (2), (4) Date: **Apr. 6, 2010**

(87) PCT Pub. No.: **WO2009/046978**

PCT Pub. Date: **Apr. 16, 2009**

(65) **Prior Publication Data**

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(30) **Foreign Application Priority Data**

Oct. 12, 2007 (EP) 07019999

(51) **Int. Cl.**
C12P 21/08 (2006.01)

(52) **U.S. Cl.**
USPC **435/69.6**; 435/70.1

(58) **Field of Classification Search**
None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,912,038	A	3/1990	Schilling, Jr. et al.
5,852,175	A	12/1998	Cummings
7,202,072	B2	4/2007	Mueller et al.
2003/0040047	A1	2/2003	Farwick et al.
2003/0096341	A1	5/2003	Mueller et al.
2007/0161079	A1	7/2007	Reiter et al.
2008/0254513	A1	10/2008	Cayli

FOREIGN PATENT DOCUMENTS

EP	0481791	4/1992
EP	0569678	11/1993
EP	0659880	6/1995
EP	1482031	12/2004
WO	8900999	2/1989
WO	89/10959	11/1989
WO	93/01296	1/1993
WO	94/25067	11/1994
WO	95/17513	6/1995
WO	99/47647	9/1999
WO	01/38557	5/2001
WO	03/076588	9/2003
WO	2004/083247	9/2004
WO	2004/087758	10/2004
WO	2005/113770	12/2005
WO	2006/072564	6/2006
WO	2007036291	4/2007
WO	2007/109052	9/2007
WO	2007/113172	10/2007

OTHER PUBLICATIONS

Dorai, H. et al., "Early prediction of instability of CHinese Hamster Ovary cell lines expressing recombinant antibodies and antibody-fusion proteins", Nov. 2011, Biotech. and Bioeng., vol. 109: pp. 1016-1030.*

Lamango, N.S. et al, Arch. Biochem. Biophys 330 (1996) 238-250.

Waldenstroem, M. et al, Gene 120 (1992) 175-181.

Moretto Nadia et al: "Conformation-Sensitive Antibodies Against Alzheimer Amyloid-Beta by Immunization wtih a Thioredoxin-Constrained B-Cell Epitope Peptide" Jour of Biolgoical Chemistry, 282:15 (2007) 11436-11445 XP002451806.

Edmonds et al, 'Development of transfection and high-producer screening protocols for the CHOK1SV cell system,' Mol Biotechnol. Oct. 2006;34(2):179-90., pp. 12.

Link et al., 'Bioprocess development for the production of a recombinant MUC1 fusion protein expressed by CHO-K1 cells in protein-free medium,' vol. 110, Issue 1, May 13, 2004, pp. 51-62., pp. 12.

(Continued)

Primary Examiner — Michael Burkhart

(57) **ABSTRACT**

The current invention reports a method for the recombinant production of a secreted heterologous immunoglobulin in a CHO cell comprising the following steps: i) providing a CHO cell, which is adapted to growth in suspension culture, adapted to growth in serum-free medium, mycoplasma free, and virus free, ii) providing a vector comprising a prokaryotic origin of replication, a first nucleic acid conferring resistance to a prokaryotic selection agent, a second nucleic acid encoding the heavy chain of said heterologous immunoglobulin, a third nucleic acid encoding the light chain of said heterologous immunoglobulin, a fourth nucleic acid conferring resistance to a eukaryotic selection agent, iii) transfecting said CHO cell, wherein said transfecting comprises a) transfecting said CHO cell with said vector comprising a fourth nucleic acid conferring resistance to a first eukaryotic selection agent, b) selecting a CHO cell by growth in cultivation medium containing said first eukaryotic selection agent, c) transfecting said selected CHO cell with said vector comprising a fourth nucleic acid conferring resistance to a second eukaryotic selection agent different to said first eukaryotic selection agent, d) selecting a CHO cell by selected growth in cultivation medium containing said first and said second eukaryotic selection agent, iv) cultivating said transfected CHO cell in a medium in the presence of said first and second eukaryotic selection agent, under conditions suitable for the expression of said second, and third nucleic acid, and v) recovering said secreted heterologous immunoglobulin from the cultivation medium.

11 Claims, 9 Drawing Sheets

(56) **References Cited**

OTHER PUBLICATIONS

Butler et al., 'Adaptation of mammalian cells to non-ammoniagenic media,' Cytotechnology 1994, vol. 15, Issue 1-3, pp. 87-94., pp. 8.
The extended European Search Report by the European Patent Office, issued on Apr. 17, 2013, in the related European application No. 12170648.5., pp. 13.
The extended European Search Report by the European Patent Office, issued on Apr. 17, 2013, in the European application No. 12191535.9., pp. 12.
Altamirano et al., 'Improvement of CHO Cell Culture Medium Formulation: Simultaneous Substitution of Glucose and Glutamine,' Biotechnol. Prog. 2000, 16, 69-75., pp. 7.
Genzel et al, 'Substitution of glutamine by pyruvate to reduce ammonia formation and growth inhibition of mammalian cells,' Biotechnol Prog. Jan.-Feb. 2005;21(1):58-69., pp. 12.
Li, J. et al., J. Immunol. Metho. 318:113-124 (2007).
(EPO Communication in Corres Appl 08836893.1 May 23, 2012).

* cited by examiner

Fig. 1

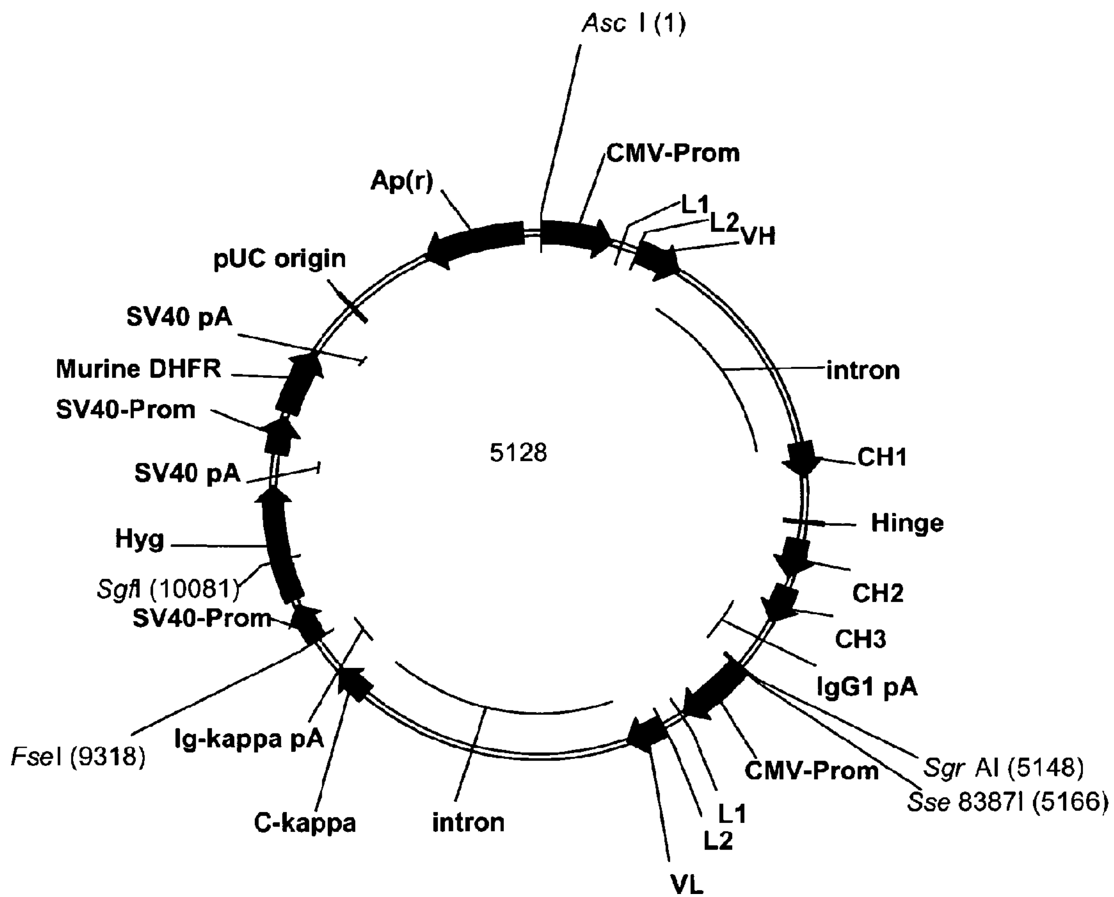


Fig. 2

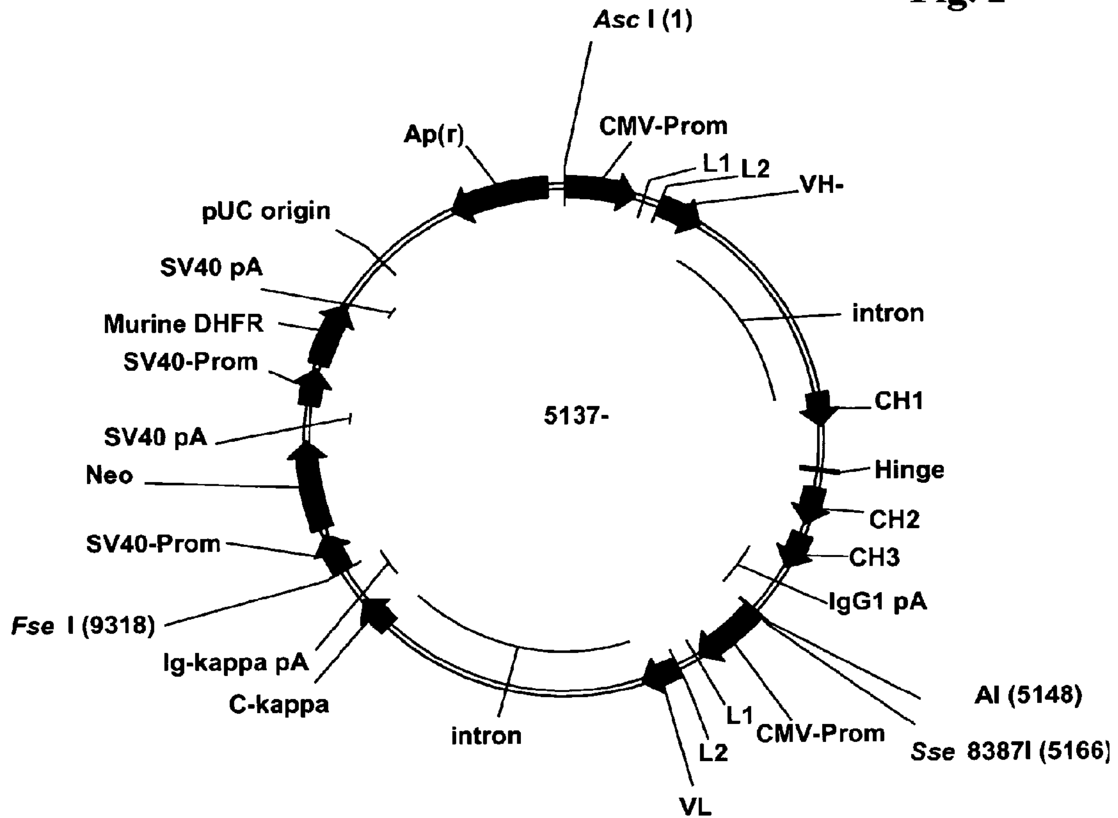


Fig. 3

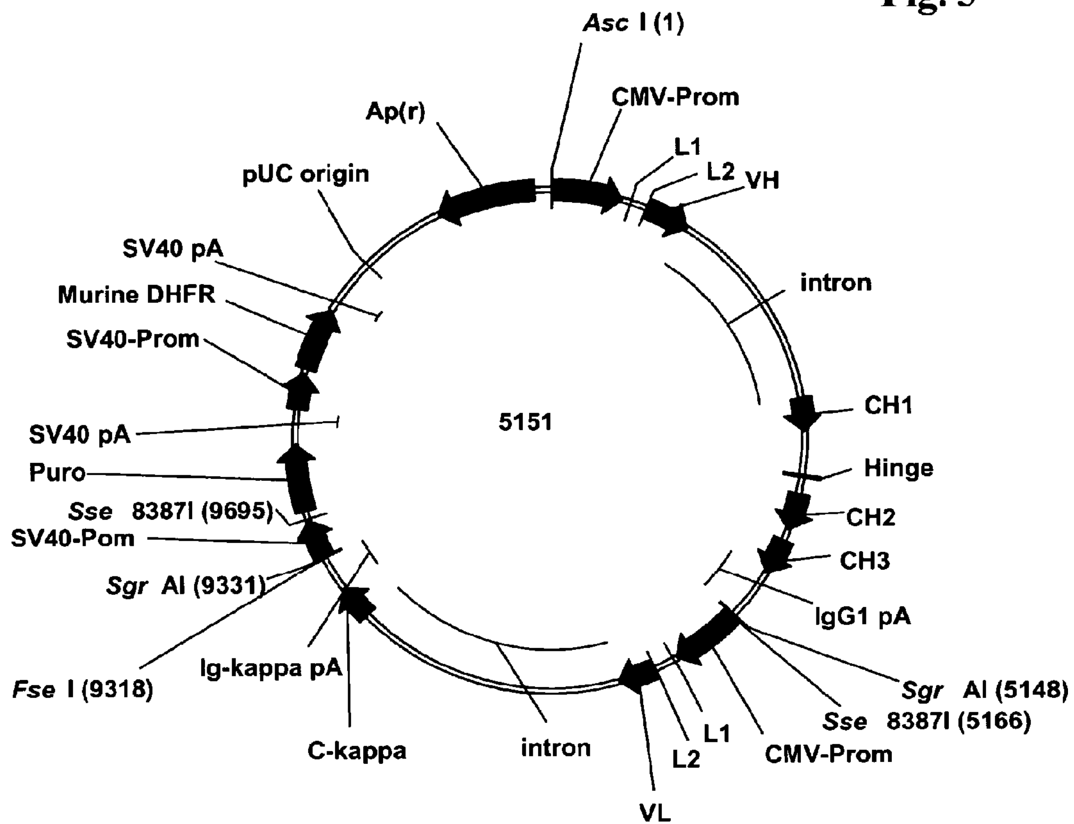


Fig. 4

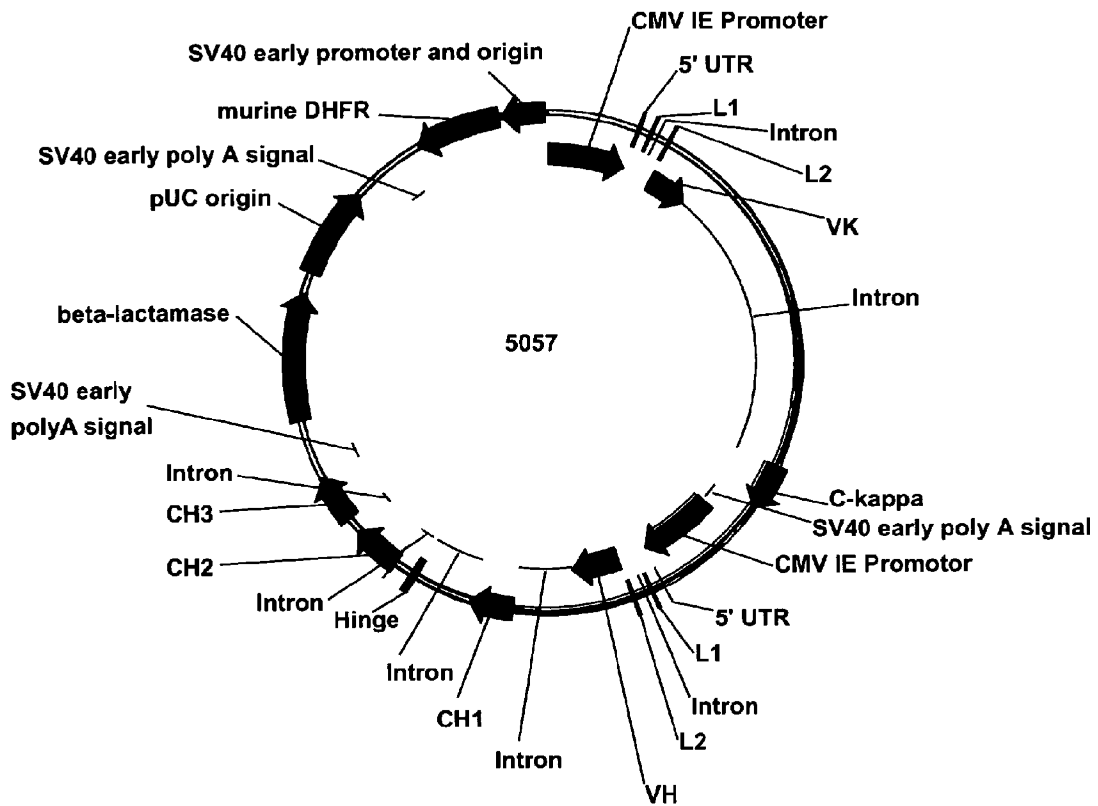


Fig. 5

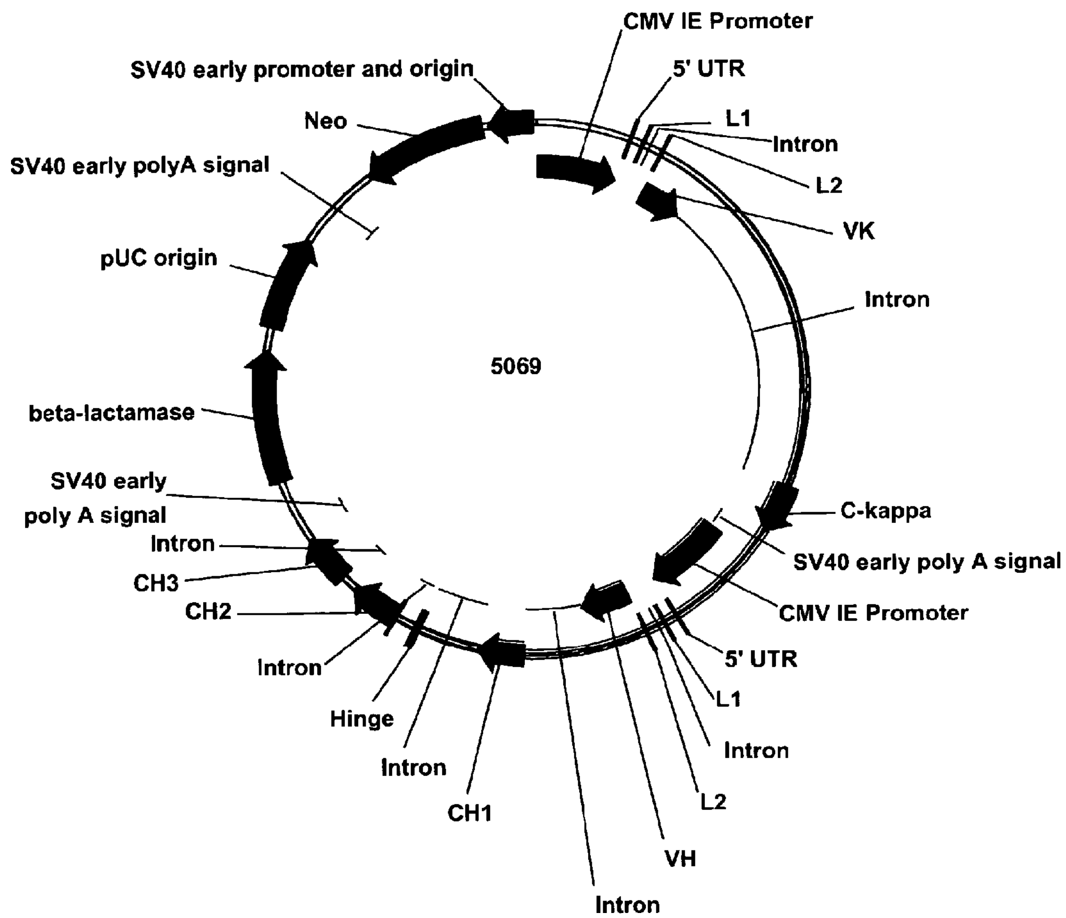
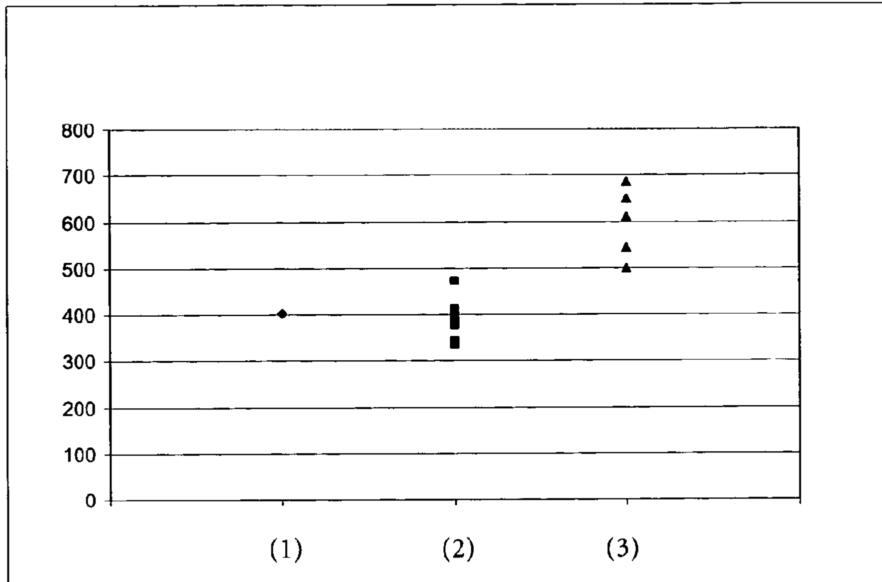


Fig. 6

(A)



(B)

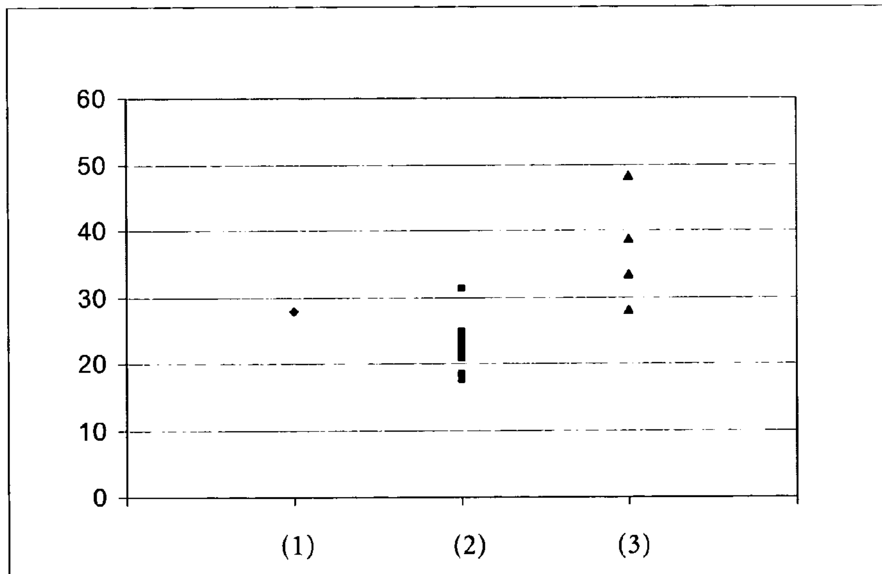


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Fig. 7

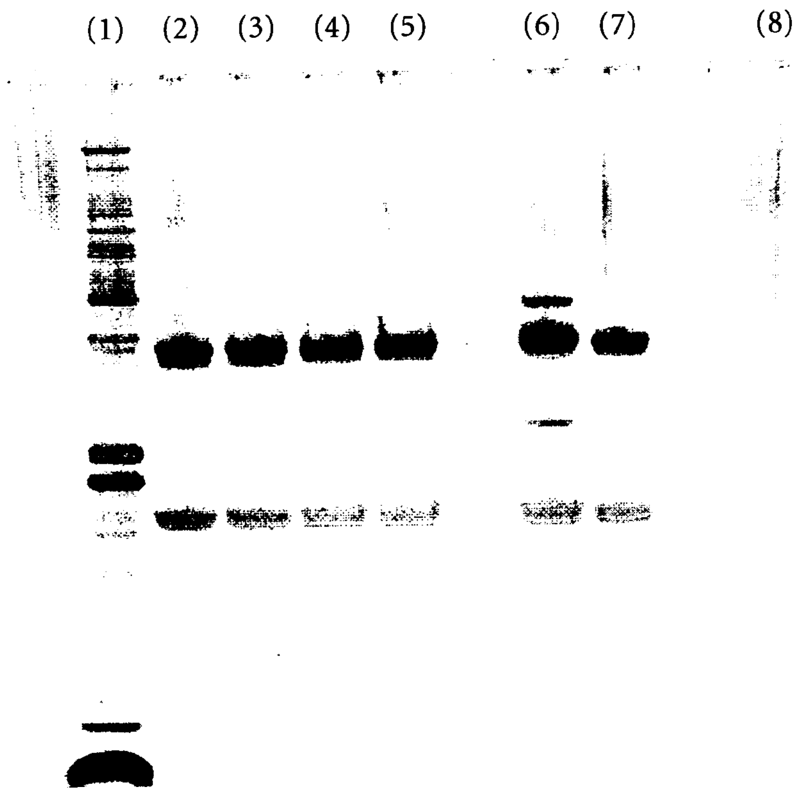


Fig. 8

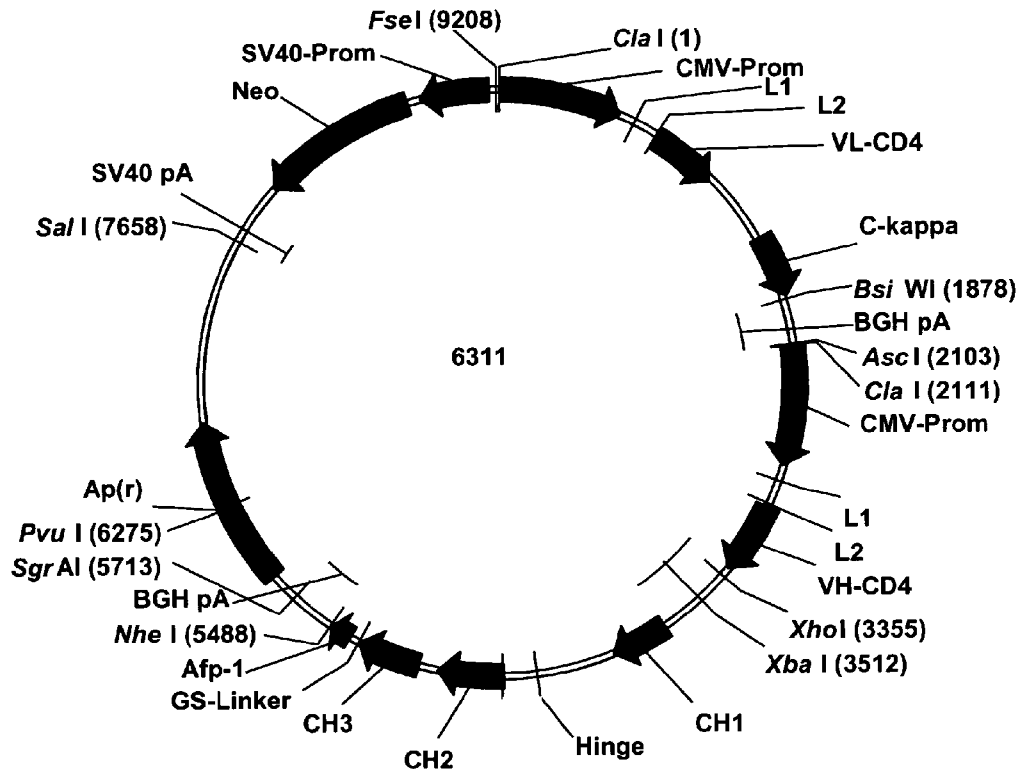
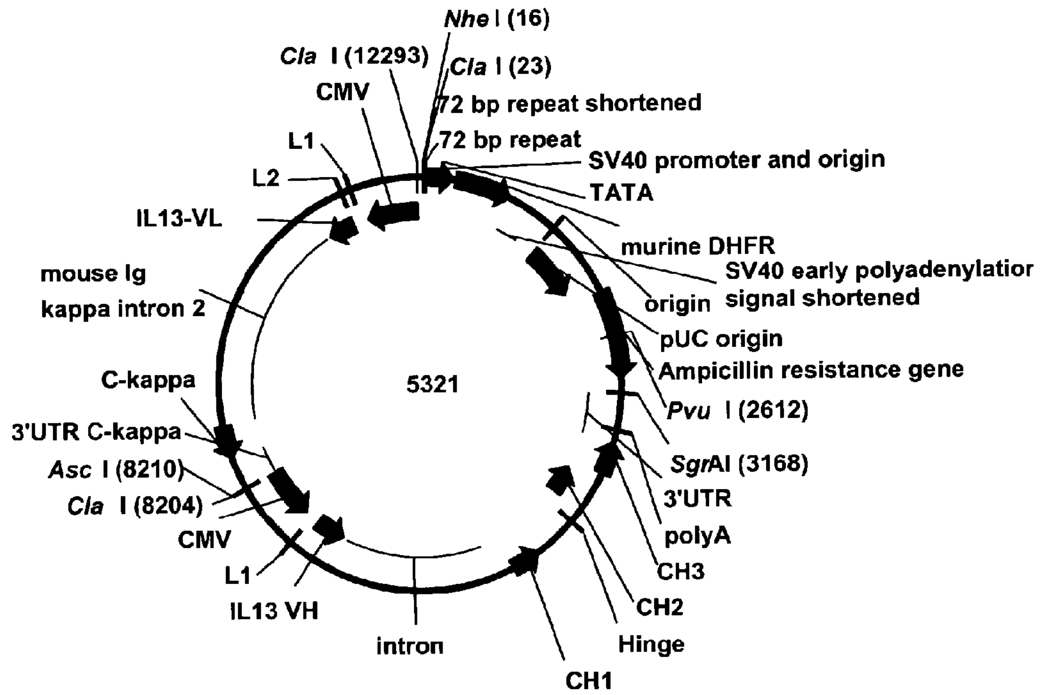


Fig. 9



PROTEIN EXPRESSION FROM MULTIPLE NUCLEIC ACIDS

This application is the National Stage of International Application No. PCT/EP2008/008523, filed Oct. 9, 2008, which claims the benefit of EP 07019999.7 filed Oct. 12, 2007, which is hereby incorporated by reference in its entirety.

The current invention is in the field of polypeptide production. More precisely it is reported the production of an immunoglobulin in a mammalian cell whereby the mammalian cell is transfected with different vectors each comprising an expression cassette for the immunoglobulin of interest.

BACKGROUND OF THE INVENTION

Expression systems for the production of recombinant polypeptides are well-known in the state of the art and are described by, e.g., Marino, M. H., Biopharm. 2 (1989) 18-33; Goeddel, D. V., et al., Methods Enzymol. 185 (1990) 3-7; Wurm, F., and Bernard, A., Curr. Opin. Biotechnol. 10 (1999) 156-159. Polypeptides for use in pharmaceutical applications are preferably produced in mammalian cells such as CHO cells, NS0 cells, SP2/0 cells, COS cells, HEK cells, BHK cells, PER.C6® cells, or the like. The essential elements of an expression plasmid are a prokaryotic plasmid propagation unit, for example for *E. coli*, comprising a prokaryotic origin of replication and a prokaryotic selection marker, an eukaryotic selection marker, and one or more expression cassettes for the expression of the structural gene(s) of interest each comprising a promoter, a structural gene, and a transcription terminator including a polyadenylation signal. For transient expression in mammalian cells a mammalian origin of replication, such as the SV40 Ori or OriP, can be included. As promoter a constitutive or inducible promoter can be selected. For optimized transcription a Kozak sequence may be included in the 5' untranslated region. For mRNA processing, in particular mRNA splicing and transcription termination, mRNA splicing signals, depending on the organization of the structural gene (exon/intron organization), may be included as well as a polyadenylation signal.

Expression of a gene is performed either as transient or as permanent expression. The polypeptide(s) of interest are in general secreted polypeptides and therefore contain an N-terminal extension (also known as the signal sequence) which is necessary for the transport/secretion of the polypeptide through the cell into the extracellular medium. In general, the signal sequence can be derived from any gene encoding a secreted polypeptide. If a heterologous signal sequence is used, it preferably is one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For secretion in yeast for example the native signal sequence of a heterologous gene to be expressed may be substituted by a homologous yeast signal sequence derived from a secreted gene, such as the yeast invertase signal sequence, alpha-factor leader (including *Saccharomyces, Kluyveromyces, Pichia*, and *Hansenula* α-factor leaders, the second described in U.S. Pat. No. 5,010,182), acid phosphatase signal sequence, or the *C. albicans* glucoamylase signal sequence (EP 0 362 179). In mammalian cell expression the native signal sequence of the protein of interest is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from secreted polypeptides of the same or related species, e.g. for immunoglobulins from human or murine origin, as well as viral secretory signal sequences, for example, the herpes simplex glycoprotein D signal sequence. The DNA fragment encoding for such a presegment is ligated in frame to the DNA fragment encoding a polypeptide of interest.

Today CHO cells are widely used for the expression of pharmaceutical polypeptides, either at small scale in the laboratory or at large scale in production processes. Due to their wide distribution and use the characteristic properties and the genetic background of CHO cells is well known. Therefore, CHO cells are approved by regulatory authorities for the production of therapeutic proteins for application to human beings.

In EP 0 569 678 are reported double transfectants of MHC genes as cellular vaccines for immunoprevention of tumor metastasis. WO 97/08342 reports an improved method for measuring the activity of a promoter sequence in a mammalian cell using a reporter gene. The use of anti-RhoA and anti-RhoC siRNAs in order to inhibit specifically RhoA or RhoC synthesis is reported in WO 2005/113770. A method for the recombinant production or expression of eukaryotic alkaline phosphatase mutant in yeast cells is reported in U.S. Pat. No. 7,202,072. WO 2001/038557 reports a method of screening multiply transformed cells using bicistronic expression of fluorescent proteins. A method for producing recombinant eukaryotic cell lines expressing multiple proteins or RNAs of interest is reported in WO 1999/47647. Systems, including methods, compositions, and kits, for transfection of cells with transfection materials using coded carriers are reported in WO 2003/076588. In U.S. Pat. No. 5,089,397 is reported an expression system for recombinant production of a desired protein comprising CHO cells transformed with a DNA sequence having the desired protein coding sequence under the control of the human metallothionein-II promoter. A method for producing recombinant proteins is reported in US 2003/0040047. Lamango et al. (Lamango, N. S., et al., Arch. Biochem. Biophys. 330 (1996) 238-250) report the dependency of the production of prohormone convertase 2 from the presence of the neuroendocrine polypeptide 7B2. The transfection of a BPV-1-based expression vector into cells harboring unintegrated replicating BPV-1 genomes is reported by Waldenstroem, M., et al., Gene 120 (1992) 175-181. U.S. Pat. No. 4,912,038 reports methods and vectors for obtaining canine and human 32K alveolar surfactant protein. In WO 89/10959 are reported recombinant DNA techniques and the expression of mammalian polypeptides in genetically engineered eukaryotic cells. A repeated co-transfer of an expression vector for human growth hormone and an expression vector for a selection marker gene is reported in DD 287531.

SUMMARY OF THE INVENTION

A first aspect of the current invention is a method for the recombinant production of a heterologous immunoglobulin which is secreted to the cultivation medium in a CHO cell comprising:

a) providing a CHO cell, which is
  - adapted to growth in suspension culture,
  - adapted to growth in serum-free medium,
  - mycoplasm free, and
  - optional virus free,

b) providing a nucleic acid comprising
  - a prokaryotic origin of replication,
  - a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,
  - a second nucleic acid sequence encoding the heavy chain of said heterologous immunoglobulin, and/or a third nucleic acid sequence encoding the light chain of said heterologous immunoglobulin,

whereby a first transfection vector is provided which comprises said provided nucleic acid, which comprises said first as well as said second and/or third nucleic acid, and an additional fourth nucleic acid sequence conferring resistance to a first eukaryotic selection agent, and

whereby a second transfection vector is provided which comprises said provided nucleic acid, which comprises the identical first as well as second and/or third nucleic acid as that/those in said provided nucleic acid contained in the first transfection vector, and an additional fourth nucleic acid sequence conferring resistance to a second eukaryotic selection agent, which is different from the fourth nucleic acid in said first transfection vector, whereby said second eukaryotic selection agent is different from said first eukaryotic selection agent,

c) transfecting said provided CHO cell and selecting said transfected CHO cell with said transfection vectors of step b), wherein said transfecting and selecting comprises the following steps in the following order:

(i) transfecting said CHO cell with said first transfection vector,

(ii) selecting a CHO cell transfected in (i) by selected growth in a cultivation medium containing said first eukaryotic selection agent to which the first transfection vector confers resistance,

(iii) transfecting said CHO cell selected in (ii) with said second transfection vector,

(iv) selecting a CHO cell transfected in (iii) by selected growth in a cultivation medium containing said first eukaryotic selection agent, to which said first transfection vector confers resistance, and containing said second eukaryotic selection agent, to which said second transfection vector confers resistance,

d) cultivating said transfected and selected CHO cell of step c) in a medium containing said first and second eukaryotic selection agent under conditions suitable for the expression of said second and/or third nucleic acid,

e) recovering said secreted heterologous immunoglobulin from the cultivation medium and thereby producing a heterologous immunoglobulin in a CHO cell, which immunoglobulin is secreted to the cultivation medium.

In one embodiment of the method according to the invention said CHO cell is a CHO K1 cell, or a CHO DG44 cell, or a CHO XL99 cell, or a CHO DXB11 cell, or a CHO DP12 cell. In another embodiment the promoter employed for the transcription of said second and third nucleic acids is different from the promoter employed for the transcription of said fourth nucleic acid. A further embodiment is that the promoter employed for the transcription of said second and third nucleic acids is the same. In one embodiment said promoter employed for the transcription of said second and third nucleic acid is the CMV promoter. In another embodiment said promoter employed for the transcription of said fourth nucleic acid is the SV40 promoter. In one embodiment said heterologous immunoglobulin is an anti-Aβ antibody. Exemplary anti-Aβ antibodies are reported e.g. in WO 2003/070760.

In one embodiment said selecting a transfected CHO cell in step c) (ii) and/or (iv) is by growth in cultivation medium without a selection agent for 10 to 72 hours followed by selected growth in a cultivation medium containing said first eukaryotic selection agent in case of (ii) or said first and second eukaryotic selection agent in case of (iv).

In still a further embodiment the codon usage of said second and third nucleic acid is optimized for the translation in CHO cells. Also an embodiment is that said second and/or third nucleic acid contains an intronic nucleic acid sequence.

Another embodiment comprises that said first transfection vector and said second transfection vector differ only in the nucleic acid conferring resistance to said eukaryotic selection agent, i.e. in said fourth nucleic acid, and are otherwise at least 95% identical based on the nucleic acid sequence. In another embodiment said transfection vectors differ each only in the nucleic acid conferring resistance to said first, second, and third eukaryotic selection agent.

In one embodiment said method further comprises:

after step b) a step b1):

b1) providing a nucleic acid comprising

a prokaryotic origin of replication,

a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,

a second nucleic acid sequence encoding the heavy chain of said heterologous immunoglobulin, and/or a third nucleic acid sequence encoding the light chain of said heterologous immunoglobulin,

whereby a third transfection vector is provided which comprises said provided nucleic acid, which comprises the identical frist as well as second and/or third nucleic acid as that/those in said provided nucleic acid contained in the first and second transfection vector, and an additional fourth nucleic acid sequence conferring resistance to a third eukaryotic selection agent, which is different from the fourth nucleic acid in said first and second transfection vector, whereby said third eukaryotic selection agent is different from said first eukaryotic selection agent and is also different from said second eukaryotic selection agent,

and further comprises after step c) (iv) the following steps (v) and (vi)

(v) transfecting said CHO cell selected in (iv) with said third transfection vector,

(vi) selecting a CHO cell transfected in (v) by selected growth in cultivation medium containing said first eukaryotic selection agent to which the first transfection vector confers resistance and said second eukaryotic selection agent to which the second transfection vector confers resistance and said third eukaryotic selection agent to which the third transfection vector confers resistance,

and further said medium for cultivating said transfected CHO cell in step d) comprises said first, second, and third eukaryotic selection agent.

In one embodiment said selecting a CHO cell transfected in step c) (vi) is by growth in cultivation medium without a selection agent for 10 to 72 hours followed by selected growth in a cultivation medium containing said first and second and third eukaryotic selection agent.

In another embodiment the method according to the invention comprises a further step

f) purifying said recombinantly produced and recovered heterologous immunoglobulin of step e) with one or more chromatographic steps.

One embodiment is that said step c) and said step d) are performed in the same medium. Still another embodiment is that said medium is a serum-free medium, or a serum-free medium supplemented with defined animal-derived components, or an animal-derived component free medium, or a protein-free medium, or a chemically defined medium, or a defined protein-free medium. In a further embodiment in said step d) is said cultivating in the presence of said eukaryotic selection agents in a volume of less than 500 liter and said cultivating is in the absence of said eukaryotic selection agents in a volume of 500 liter or more, whereby said recovering said secreted heterologous immunoglobulin is from the

cultivation medium without said eukaryotic selection agents. In a further embodiment said cultivating in said step d) is comprising sequential cultivations each with increasing cultivation volume up to a preset final cultivation volume, whereby the cultivations are performed in the presence of said eukaryotic selection agents up to a cultivation volume of 1% (v/v) of the cultivation volume of the final cultivation and in the absence of said eukaryotic selection agents in a cultivation volume of more than 1% (v/v) of the cultivation volume of the final cultivation.

The productivity of said CHO cells is in one embodiment over 40 generations not less than 70% and not more than 130% of the productivity after 10 generations of cultivation as split-batch cultivation. In another embodiment is the productivity of said CHO cells over 60 generations not less than 50% and not more than 150% of the productivity after 10 generations of cultivation as split-batch cultivation. In still a further embodiment is the productivity of said CHO cell at least 1.5 g/l of said heterologous immunoglobulin within 21 days as fed-batch cultivation.

A second aspect of the current invention is a CHO cell obtainable with the following method:

a) providing a CHO cell, which is
   - adapted to growth in suspension culture,
   - adapted to growth in serum-free medium,
   - mycoplasma free, and
   - optional virus free,
b) providing a nucleic acid comprising
   - a prokaryotic origin of replication,
   - a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,
   - a second nucleic acid sequence encoding the heavy chain of a heterologous immunoglobulin, and/or a third nucleic acid sequence encoding the light chain of a heterologous immunoglobulin,
   - whereby a first transfection vector is provided which comprises said provided nucleic acid, which comprises said frist as well as second and/or third nucleic acid, and an additional fourth nucleic acid sequence conferring resistance to a first eukaryotic selection agent, and
   - whereby a second transfection vector is provided which comprises said provided nucleic acid, which comprises the identical frist as well as second and/or third nucleic acid as that/those in said provided nucleic acid contained in the first transfection vector, and an additional fourth nucleic acid sequence conferring resistance to a second eukaryotic selection agent, which is different from the fourth nucleic acid in said first transfection vector, whereby said second eukaryotic selection agent is different from said first eukaryotic selection agent,
c) transfecting said CHO cell, wherein said transfecting comprises the following steps in the following order:
   - (i) transfecting said CHO cell with said first transfection vector,
   - (ii) selecting a CHO cell transfected in (i) by selected growth in cultivation medium containing a first eukaryotic selection agent to which the first transfection vector confers resistance,
   - (iii) transfecting said CHO cell selected in (ii) with said second transfection vector,
   - (iv) selecting a CHO cell transfected in (iii) by selected growth in cultivation medium containing said first eukaryotic selection agent to which the first transfection vector confers resistance and said second eukaryotic selection agent to which the second transfection vector confers resistance.

DETAILED DESCRIPTION OF THE INVENTION

Methods and techniques known to a person skilled in the art, which are useful for carrying out the current invention, are described e.g. in Ausubel, F. M., ed., Current Protocols in Molecular Biology, Volumes I to III (1997), Wiley and Sons; Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

General chromatographic methods and their use are known to a person skilled in the art. See for example, Chromatography, 5th edition, Part A: Fundamentals and Techniques, Heftmann, E. (ed), Elsevier Science Publishing Company, New York, (1992); Advanced Chromatographic and Electromigration Methods in Biosciences, Deyl, Z. (ed.), Elsevier Science BV, Amsterdam, The Netherlands, (1998); Chromatography Today, Poole, C. F., and Poole, S. K., Elsevier Science Publishing Company, New York, (1991); Scopes, Protein Purification: Principles and Practice (1982); Sambrook, J., et al. (ed), Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; or Current Protocols in Molecular Biology, Ausubel, F. M., et al. (eds), John Wiley & Sons, Inc., New York.

For the purification of recombinantly produced heterologous immunoglobulins often a combination of different column chromatography steps is employed. Generally a Protein A affinity chromatography is followed by one or two additional separation steps. The final purification step is a so called "polishing step" for the removal of trace impurities and contaminants like aggregated immunoglobulins, residual HCP (host cell protein), DNA (host cell nucleic acid), viruses, or endotoxins. For this polishing step often an anion exchange material in a flow-through mode is used.

Different methods are well established and widespread used for protein recovery and purification, such as affinity chromatography with microbial proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, azaarenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and electrophoretical methods (such as gel electrophoresis, capillary electrophoresis) (Vijayalakshmi, M. A., Appl. Biochem. Biotech. 75 (1998) 93-102).

The term "amino acid" as used within this application denotes the group of carboxy α-amino acids, which directly or in form of a precursor can be encoded by a nucleic acid. The individual amino acids are encoded by nucleic acids consisting of three nucleotides, so called codons or base-triplets. Each amino acid is encoded by at least one codon. The encoding of the same amino acid by different codons is known as "degeneration of the genetic code". The term "amino acid" as used within this application denotes the naturally occurring carboxy α-amino acids and is comprising alanine (three letter code: ala, one letter code: A), arginine (arg, R), asparagine (asn, N), aspartic acid (asp, D), cysteine (cys, C), glutamine (gln, Q), glutamic acid (glu, E), glycine (gly, G), histidine (his, H), isoleucine (ile, I), leucine (leu, L), lysine (lys, K), methionine (met, M), phenylalanine (phe, F), proline (pro, P), serine (ser, S), threonine (thr, T), tryptophan (trp, W), tyrosine (tyr, Y), and valine (val, V).

A "nucleic acid" or a "nucleic acid sequence", which terms are used interchangeably within this application, refers to a polymeric molecule consisting of individual nucleotides (also called bases) a, c, g, and t (or u in RNA), for example to DNA, RNA, or modifications thereof. This polynucleotide molecule can be a naturally occurring polynucleotide molecule or a synthetic polynucleotide molecule or a combination of one or more naturally occurring polynucleotide molecules with one or more synthetic polynucleotide molecules. Also encompassed by this definition are naturally occurring polynucleotide molecules in which one or more nucleotides are changed (e.g. by mutagenesis), deleted, or added. A nucleic acid can either be isolated, or integrated in another nucleic acid, e.g. in an expression cassette, a plasmid, or the chromosome of a host cell. A nucleic acid is characterized by its nucleic acid sequence consisting of individual nucleotides.

To a person skilled in the art procedures and methods are well known to convert an amino acid sequence, e.g. of a polypeptide, into a corresponding nucleic acid sequence encoding this amino acid sequence. Therefore, a nucleic acid is characterized by its nucleic acid sequence consisting of individual nucleotides and likewise by the amino acid sequence of a polypeptide encoded thereby.

A "polypeptide" is a polymer consisting of amino acids joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 20 amino acid residues may be referred to as "peptides", whereas molecules consisting of two or more polypeptides or comprising one polypeptide of more than 100 amino acid residues may be referred to as "proteins". A polypeptide may also comprise non-amino acid components, such as carbohydrate groups, metal ions, or carboxylic acid esters. The non-amino acid components may be added by the cell, in which the polypeptide is expressed, and may vary with the type of cell. Polypeptides are defined herein in terms of their amino acid backbone structure or the nucleic acid encoding the same. Additions such as carbohydrate groups are generally not specified, but may be present nonetheless.

The term "immunoglobulin" encompasses the various forms of immunoglobulin structures including complete immunoglobulins and immunoglobulin conjugates. The immunoglobulin employed in the current invention is preferably a human antibody, or a humanized antibody, or a chimeric antibody, or a T cell antigen depleted antibody (see e.g. WO 98/33523, WO 98/52976, and WO 00/34317). Genetic engineering of antibodies is e.g. described in Morrison, S. L., et al., Proc. Natl. Acad. Sci. USA 81 (1984) 6851-6855; U.S. Pat. Nos. 5,202,238 and 5,204,244; Riechmann, L., et al., Nature 332 (1988) 323-327; Neuberger, M. S., et al., Nature 314 (1985) 268-270; Lonberg, N., Nat. Biotechnol. 23 (2005) 1117-1125. Immunoglobulins may exist in a variety of formats, including, for example, Fv, Fab, and $F(ab)_2$ as well as single chains (scFv) or diabodies (e.g. Huston, J. S., et al., Proc. Natl. Acad. Sci. USA 85 (1988) 5879-5883; Bird, R. E., et al., Science 242 (1988) 423-426; in general, Hood et al., Immunology, Benjamin N.Y., 2nd edition (1984); and Hunkapiller, T. and Hood, L., Nature 323 (1986) 15-16).

The term "complete immunoglobulin" denotes an immunoglobulin which comprises two so called light chains and two so called heavy chains. Each of the heavy and light chains of a complete immunoglobulin contains a variable domain (variable region) (generally the amino terminal portion of the polypeptide chain) comprising binding regions that are able to interact with an antigen. Each of the heavy and light chains of a complete immunoglobulin comprises a constant region (generally the carboxyl terminal portion). The constant region of the heavy chain mediates the binding of the antibody i) to cells bearing a Fc gamma receptor (FcγR), such as phagocytic cells, or ii) to cells bearing the neonatal Fc receptor (FcRn) also known as Brambell receptor. It also mediates the binding to some factors including factors of the classical complement system such as component (C1q). The variable domain of an immunoglobulin's light or heavy chain in turn comprises different segments, i.e. four framework regions (FR) and three hypervariable regions (CDR).

The term "immunoglobulin conjugate" denotes a polypeptide comprising at least one domain of an immunoglobulin heavy or light chain conjugated via a peptide bond to a further polypeptide. The further polypeptide is a non-immunoglobulin peptide, such as a hormone, or growth receptor, or antifusogenic peptide, or complement factor, or the like. Exemplary immunoglobulin conjugates are reported in WO 2007/045463.

The term "heterologous immunoglobulin" denotes an immunoglobulin which is not naturally produced by a mammalian cell or the host cell. The immunoglobulin produced according to the method of the invention is produced by recombinant means. Such methods are widely known in the state of the art and comprise protein expression in eukaryotic cells with subsequent recovery and isolation of the heterologous immunoglobulin, and usually purification to a pharmaceutically acceptable purity. For the production, i.e. expression, of an immunoglobulin a nucleic acid encoding the light chain and a nucleic acid encoding the heavy chain are inserted each into an expression cassette by standard methods. Nucleic acids encoding immunoglobulin light and heavy chains are readily isolated and sequenced using conventional procedures. Hybridoma cells can serve as a source of such nucleic acids. The expression cassettes may be inserted into an expression plasmid(s), which is (are) then transfected into host cells, which do not otherwise produce immunoglobulins. Expression is performed in appropriate prokaryotic or eukaryotic host cells and the immunoglobulin is recovered from the cells after lysis or from the culture supernatant.

An "isolated polypeptide" is a polypeptide that is essentially free from contaminating cellular components, such as carbohydrate, lipid, or other proteinaceous impurities associated with the polypeptide in nature. Typically, a preparation of isolated polypeptide contains the polypeptide in a highly purified form, i.e. at least about 80% pure, at least about 90% pure, at least about 95% pure, greater than 95% pure, or greater than 99% pure. One way to show that a particular protein preparation contains an isolated polypeptide is by the appearance of a single band following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the protein preparation and Coomassie Brilliant Blue staining of the gel. However, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

"Heterologous DNA" or "heterologous polypeptide" refers to a DNA molecule or a polypeptide, or a population of DNA molecules or a population of polypeptides, that do not exist naturally within a given host cell. DNA molecules heterologous to a particular host cell may contain DNA derived from the host cell species (i.e. endogenous DNA) so long as that host DNA is combined with non-host DNA (i.e. exogenous DNA). For example, a DNA molecule containing a non-host DNA segment encoding a polypeptide operably linked to a host DNA segment comprising a promoter is considered to be a heterologous DNA molecule. Conversely, a heterologous DNA molecule can comprise an endogenous structural gene operably linked with an exogenous promoter.

A peptide or polypeptide encoded by a non-host DNA molecule is a "heterologous" peptide or polypeptide.

The term "cell" or "host cell" refers to a cell into which a nucleic acid, e.g. encoding a heterologous polypeptide, can be or is transfected. The term "cell" includes both prokaryotic cells, which are used for propagation of plasmids, and eukaryotic cells, which are used for the expression of a nucleic acid and production of the encoded polypeptide. In one embodiment, the eukaryotic cells are mammalian cells. In another embodiment the mammalian cell is a CHO cell, preferably a CHO K1 cell (ATCC CCL-61 or DSM ACC 110), or a CHO DG44 cell (also known as CHO-DHFR[−], DSM ACC 126), or a CHO XL99 cell, a CHO-T cell (see e.g. Morgan, D., et al., Biochemistry 26 (1987) 2959-2963), or a CHO-S cell, or a Super-CHO cell (Pak, S. C. O., et al. Cytotechnology. 22 (1996) 139-146). If these cells are not adapted to growth in serum-free medium or in suspension an adaptation prior to the use in the current method is to be performed. As used herein, the expression "cell" includes the subject cell and its progeny. Thus, the words "transformant" and "transformed cell" include the primary subject cell and cultures derived there from without regard for the number of transfers or subcultivations. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

The term "expression" as used herein refers to transcription and/or translation processes occurring within a cell. The level of transcription of a nucleic acid sequence of interest in a cell can be determined on the basis of the amount of corresponding mRNA that is present in the cell. For example, mRNA transcribed from a sequence of interest can be quantitated by RT-PCR or by Northern hybridization (see Sambrook, et al., 1989, supra). Polypeptides encoded by a nucleic acid of interest can be quantitated by various methods, e.g. by ELISA, by assaying for the biological activity of the polypeptide, or by employing assays that are independent of such activity, such as Western blotting or radioimmunoassay, using immunoglobulins that recognize and bind to the polypeptide (see Sambrook, et al., 1989, supra).

An "expression cassette" refers to a construct that contains the necessary regulatory elements, such as promoter and polyadenylation site, for expression of at least the contained nucleic acid in a cell.

A "transfection vector" is a nucleic acid (also denoted as nucleic acid molecule) providing all required elements for the expression of the in the transfection vector comprised coding nucleic acids/structural gene(s) in a host cell. A transfection vector comprises a prokaryotic plasmid propagation unit, e.g. for *E. coli*, in turn comprising a prokaryotic origin of replication, and a nucleic acid conferring resistance to a prokaryotic selection agent, further comprises the transfection vector one or more nucleic acid(s) conferring resistance to an eukaryotic selection agent, and one or more nucleic acid encoding a polypeptide of interest. Preferably are the nucleic acids conferring resistance to a selection agent and the nucleic acid(s) encoding a polypeptide of interest placed each within an expression cassette, whereby each expression cassette comprises a promoter, a coding nucleic acid, and a transcription terminator including a polyadenylation signal. Gene expression is usually placed under the control of a promoter, and such a structural gene is said to be "operably linked to" the promoter. Similarly, a regulatory element and a core promoter are operably linked if the regulatory element modulates the activity of the core promoter.

A "promoter" refers to a polynucleotide sequence that controls transcription of a gene/structural gene or nucleic acid sequence to which it is operably linked. A promoter includes signals for RNA polymerase binding and transcription initiation. The promoter(s) used will be functional in the cell type of the host cell in which expression of the selected sequence is contemplated. A large number of promoters including constitutive, inducible and repressible promoters from a variety of different sources, are well known in the art (and identified in databases such as GenBank) and are available as or within cloned polynucleotides (from, e.g., depositories such as ATCC as well as other commercial or individual sources). A "promoter" comprises a nucleotide sequence that directs the transcription of an operably linked structural gene. Typically, a promoter is located in the 5' non-coding or untranslated region of a gene, proximal to the transcriptional start site of a structural gene. Sequence elements within promoters that function in the initiation of transcription are often characterized by consensus nucleotide sequences. These promoter elements include RNA polymerase binding sites, TATA sequences, CAAT sequences, differentiation-specific elements (DSEs; McGehee, R. E., et al., Mol. Endocrinol. 7 (1993) 551-560), cyclic AMP response elements (CREs), serum response elements (SREs; Treisman, R., Seminars in Cancer Biol. 1 (1990) 47-58), glucocorticoid response elements (GREs), and binding sites for other transcription factors, such as CRE/ATF (O'Reilly, M. A., et al., J. Biol. Chem. 267 (1992) 19938-19943), AP2 (Ye, J., et al., J. Biol. Chem. 269 (1994) 25728-25734), SP1, cAMP response element binding protein (CREB; Loeken, M. R., Gene Expr. 3 (1993) 253-264) and octamer factors (see, in general, Watson et al., eds., Molecular Biology of the Gene, 4th ed. (The Benjamin/Cummings Publishing Company, Inc. 1987), and Lemaigre, F. P. and Rousseau, G. G., Biochem. J. 303 (1994) 1-14). Among the eukaryotic promoters that have been identified as strong promoters for high-level expression are the SV40 early promoter, adenovirus major late promoter, mouse metallothionein-I promoter, Rous sarcoma virus long terminal repeat, Chinese hamster elongation factor 1 alpha (CHEF-1, see e.g. U.S. Pat. No. 5,888,809), human EF-1 alpha, ubiquitin, and human cytomegalovirus immediate early promoter (CMV IE).

The "promoter" can be constitutive or inducible. An enhancer (i.e., a cis-acting DNA element that acts on a promoter to increase transcription) may be necessary to function in conjunction with the promoter to increase the level of expression obtained with a promoter alone, and may be included as a transcriptional regulatory element. Often, the polynucleotide segment containing the promoter will include enhancer sequences as well (e.g., CMV or SV40).

An "enhancer", as used herein, refers to a polynucleotide sequence that enhances transcription of a gene or coding sequence to which it is operably linked. Unlike promoters, enhancers are relatively orientation and position independent and have been found 5' or 3' (Lusky, M., et al., Mol. Cell Bio., 3 (1983) 1108-1122) to the transcription unit, within an intron (Banerji, J., et al., Cell, 33 (1983) 729-740) as well as within the coding sequence itself (Osborne, T. F., et al., Mol. Cell. Bio., 4 (1984) 1293-1305). Therefore, enhancers may be placed upstream or downstream from the transcription initiation site or at considerable distances from the promoter, although in practice enhancers may overlap physically and functionally with promoters. A large number of enhancers, from a variety of different sources are well known in the art (and identified in databases such as GenBank) and are available as or within cloned polynucleotide sequences (from, e.g., depositories such as the ATCC as well as other commercial or individual sources). A number of polynucleotides comprising promoter sequences (such as the commonly-used CMV promoter) also comprise enhancer sequences. For example, all of

the strong promoters listed above may also contain strong enhancers (see e.g. Bendig, M., M., Genetic Engineering 7 (Academic Press, 1988) 91-127).

A "nucleic acid conferring resistance to a selection agent" is a nucleic acid that allows cells carrying it to be specifically selected for or against, in the presence of a selection agent. Such a nucleic acid is also denoted as selection marker. Typically, a selection marker will confer resistance to a selection agent (drug) or compensate for a metabolic or catabolic defect in the host cell. A selection marker can be positive, negative, or bifunctional. A useful positive selection marker is an antibiotic resistance gene. This selection marker allows cells transformed therewith to be positively selected for in the presence of the corresponding selection agent, i.e. under selected growth in the presence e.g. of the corresponding antibiotic. A non-transformed cell is not capable to grow or survive under the selective growth conditions, i.e. in the presence of the selection agent, in culture. Positive selection markers allow selection for cells carrying the marker, whereas negative selection markers allow cells carrying the marker to be selectively eliminated. Eukaryotic selection markers include, e.g., the genes for aminoglycoside phosphotransferase (APH) (conferring resistance to the selection agents such as e.g. hygromycin (hyg), neomycin (neomycin phosphotransferase II, neo), and G418), dihydrofolate reductase (DHFR) (conferring resistance to the selection agent methotrexate), thymidine kinase (tk), glutamine synthetase (GS), asparagine synthetase, tryptophan synthetase (conferring resistance to the selection agent indole), histidinol dehydrogenase (conferring resistance to the selection agent histidinol D), cytidine deaminase, adenosine deaminase and nucleic acids conferring resistance to puromycin, bleomycin, phleomycin, chloramphenicol, Zeocin, and mycophenolic acid. Further selection marker nucleic acids are reported e.g. in WO 92/08796 and WO 94/28143. Prokaryotic selection markers include, e.g. the beta-lactamase gene (conferring resistance to the selection agent ampicillin).

Expression of a gene is performed either as transient or as permanent expression. The polypeptide(s) of interest are in general secreted polypeptides and therefore contain an N-terminal extension (also known as the signal sequence) which is necessary for the transport/secretion of the polypeptide through the cell wall into the extracellular medium. In general, the signal sequence can be derived from any gene encoding a secreted polypeptide. If a heterologous signal sequence is used, it preferably is one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For secretion in yeast for example the native signal sequence of a heterologous gene to be expressed may be substituted by a homologous yeast signal sequence derived from a secreted gene, such as the yeast invertase signal sequence, alpha-factor leader (including *Saccharomyces, Kluyveromyces, Pichia*, and *Hansenula* α-factor leaders, the second described in U.S. Pat. No. 5,010,182), acid phosphatase signal sequence, or the *C. albicans* glucoamylase signal sequence (EP 0 362 179). In mammalian cell expression the native signal sequence of the protein of interest is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from secreted polypeptides of the same or related species, e.g. for immunoglobulins from human or murine origin, as well as viral secretory signal sequences, for example, the herpes simplex glycoprotein D signal sequence. The DNA fragment encoding for such a presegment is ligated in frame, i.e. operably linked, to the DNA fragment encoding a polypeptide of interest.

The first aspect of the current invention is a method for the recombinant production of a secreted heterologous immunoglobulin in a CHO cell which comprises:

a) providing a CHO cell, which is adapted to growth in suspension culture, adapted to growth in serum-free medium, and mycoplasma free;
b) providing a transfection vector, which comprises the following elements:
   a prokaryotic origin of replication,
   a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,
   a second nucleic acid sequence encoding the heavy chain of said heterologous immunoglobulin and a third nucleic acid sequence encoding the light chain of said heterologous immunoglobulin,
   a fourth nucleic acid sequence conferring resistance to a eukaryotic selection agent,
   whereby each of said first to fourth nucleic acid sequence is contained in an expression cassette,
c) transfecting and selecting said CHO cell, wherein said transfecting and selecting comprises the following steps in the following order:
   (i) transfecting said CHO cell with a transfection vector comprising said first to third nucleic acid and a fourth nucleic acid sequence conferring resistance to a first eukaryotic selection agent,
   (ii) selecting a CHO cell transfected in (i) by selected growth in cultivation medium containing said first eukaryotic selection agent,
   (iii) transfecting said CHO cell selected in (ii) with a transfection vector comprising said first to third nucleic acid and a fourth nucleic acid sequence different from that in the transfection vector used in (i) conferring resistance to a second eukaryotic selection agent different to said first eukaryotic selection agent,
   (iv) selecting a CHO cell transfected in (iii) by selected growth in cultivation medium containing said first and said second eukaryotic selection agent,
d) cultivating said transfected and selected CHO cell of step c) in a cultivation medium containing said first and second eukaryotic selection agent, under conditions suitable for the expression of said second, and third nucleic acid,
e) recovering said secreted heterologous immunoglobulin from the cultivation medium and thereby recombinantly producing a heterologous immunoglobulin.

The method according to the invention is suited for the production of a secreted heterologous immunoglobulin in large scale, i.e. industrially. The cultivation of a cell for the production of a desired polypeptide in large scale generally consists of a sequence of individual cultivations, wherein all cultivations except the final, i.e. the large scale, cultivation, i.e. the last one in the sequence, are performed until a certain cell density is reached in the culture vessel. If the predetermined cell density is reached the entire cultivation or a fraction thereof is used to inoculate the next cultivation vessel, which has a larger volume, up to 1000 times the volume of the preceding cultivation. All cultivations which serve as a basis for at least one further cultivation in a larger volume are denoted as seed train fermentations. Only in the large scale cultivation, i.e. in the cultivation which is not intended to serve as the basis for a further cultivation in a larger volume, which is also denoted as main fermentation, is the endpoint of the cultivation determined depending on the concentration of the produced secreted heterologous immunoglobulin in the cultivation medium. The term "large scale" as used within this application denotes the final cultivation of an industrial production process. Preferably a large scale cultivation is

performed at a volume of at least 100 l, more preferably of at least 500 l, most preferably of at least 1000 l up to a volume of 20,000 l. In one embodiment the final, i.e. large scale, cultivation medium does not contain a eukaryotic selection agent.

In one embodiment the cultivation of said transfected CHO cell is performed in the presence of said eukaryotic selection agent in a volume of less than 500 liter and the cultivation of said transfected CHO cell is performed in the absence of said eukaryotic selection agents in a volume of 500 liter or more and that said recovering said secreted heterologous immunoglobulin is from the cultivation medium without said eukaryotic selection agents. In a further embodiment the cultivation is comprising sequential cultivations with increasing cultivation volume up to a final cultivation volume, whereby the cultivations are performed in the presence of said eukaryotic selection agents up to a cultivation volume of 1% (v/v) of the cultivation volume of the final or main cultivation and in the absence of all of said eukaryotic selection agents in a cultivation volume of more than 1% (v/v) of the cultivation volume of the final cultivation. In a further embodiment said cultivation is comprising sequential seed train cultivations with increasing cultivation volume, whereby each of the seed train cultivations is performed in the presence of said eukaryotic selection agents and the main fermentation is performed in the absence of all of said eukaryotic selection agents. In one embodiment the cultivation of said transfected CHO cell is performed in the presence of said eukaryotic selection agent in the seed train fermentations and the cultivation of said transfected CHO cell is performed in the absence of said eukaryotic selection agents in the main fermentation and that said recovering said secreted heterologous immunoglobulin is from the main cultivation medium not containing said eukaryotic selection agents. In these embodiments the eukaryotic selection agents are added during the growth phase and omitted during the production phase of said CHO cell. The term “production phase” denotes the cultivation of a CHO cell in a large volume, i.e. the main fermentation, after which the produced heterologous immunoglobulin is recovered.

In another embodiment of the method according to the invention the productivity of said CHO cell is over 40 generations not less than 70% and not more than 130% of the productivity after 10 generations of cultivation as split-batch cultivation. In an embodiment the productivity of said CHO cells is over 60 generations not less than 50% and not more than 150% of the productivity after 10 generations of cultivation as split-batch cultivation. The productivity of said CHO cell is at least 1.5 g/l of said heterologous immunoglobulin within 21 days as fed-batch cultivation in another embodiment. In one embodiment the specific productivity of the CHO cell obtained with the method according to the invention is more than 1 $\mu g/10^6$ cells/d, more than 5 $\mu g/10^6$ cells/d, or more than 10 $\mu g/10^6$ cells/d. In one embodiment the secreted heterologous immunoglobulin is a completely processed secreted heterologous immunoglobulin. The term “completely processed secreted heterologous immunoglobulin” denotes an immunoglobulin i) which is secreted to the cultivation medium and whose signal sequences has been cleaved, ii) which comprises an antigen binding region, iii) which has secondary modifications, such as attached saccharides or polysaccharides, and/or correctly formed disulfide bonds.

In one embodiment of the invention the heterologous immunoglobulin is an anti-Aβ antibody. In another embodiment the heavy chain variable domain of said anti-Aβ antibody comprises a CDR3 with an amino acid sequence selected from SEQ ID NO: 1, 2, or 3. In a further embodiment the light chain variable domain of said anti-Aβ antibody comprises a CDR3 with an amino acid sequence selected from SEQ ID NO: 4, 5, or 6. In a further embodiment said anti-Aβ antibody comprises a heavy chain variable domain with an amino acid sequence selected from SEQ ID NO: 7, 8, or 9. In still a further embodiment said anti-Aβ antibody comprises a light chain variable domain with an amino acid sequence selected from SEQ ID NO: 10, 11, or 12.

In one embodiment of the invention the heterologous immunoglobulin is an anti-P-Selectin antibody. In a further embodiment said anti-P-Selectin antibody comprises a heavy chain variable domain with an amino acid sequence selected from SEQ ID NO: 13, 14, or 15. In still a further embodiment said anti-P-Selectin antibody comprises a light chain variable domain with an amino acid sequence selected from SEQ ID NO: 16, 17, or 18.

In one embodiment of the invention the heterologous immunoglobulin is an anti-IL-13Rα antibody. In a further embodiment said anti-IL-13Rα antibody comprises a heavy chain variable domain with an amino acid sequence selected from SEQ ID NO: 19, 20, 21, 22, or 23. In still a further embodiment said anti-IL-13Rα antibody comprises a light chain variable domain with an amino acid sequence selected from SEQ ID NO: 24, 25, 26, 27, or 28.

In one embodiment of the invention the heterologous immunoglobulin is an anti-CD4 antibody-conjugate. In another embodiment the heavy chain variable domain of said anti-CD4 antibody in said conjugate comprises a CDR3 with an amino acid sequence selected from SEQ ID NO: 29, 30, or 31. In a further embodiment the light chain variable domain of said anti-CD4 antibody in said conjugate comprises a CDR3 with an amino acid sequence selected from SEQ ID NO: 32, 33, or 34. In a further embodiment said anti-CD4 antibody in said conjugate comprises a heavy chain variable domain with an amino acid sequence selected from SEQ ID NO: 35, 36, or 37. In still a further embodiment said anti-CD4 antibody in said conjugate comprises a light chain variable domain with an amino acid sequence selected from SEQ ID NO: 38, 39, or 40.

A mammalian cell usable for the large scale production of therapeutics, i.e. polypeptides intended for the use in humans, has to fulfill distinct criteria. Amongst others are these that it has to be cultivatable in serum-free, preferably in non-defined mammal-derived components free medium, or in a serum-free medium supplemented with defined mammal-derived components. Serum is a mixture of multitude of compounds. Normally bovine serum has been used for the cultivation of mammalian cells. With the arising problem of transmissible diseases from one species to another the use of serum and other non-defined mammal-derived compounds has to be avoided. The term “non-defined mammal-derived compound” as used within this application denotes compounds which are derived from a mammal, especially preferred from a cow, a pig, a sheep, or a lamb, and whose composition can be specified to less than 80%, preferably to less than 90% (w/w). A “defined mammal-derived compound” is a compound that is obtained from a mammal, especially preferred from a cow, a pig, a sheep, or a lamb, and whose composition can be specified to more than 95% (w/w), preferably to more than 98% (w/w), most preferably to more then 99% (w/w). An example of a defined mammal-derived compound is cholesterol from ovine wool, and galactose from bovine milk. In one embodiment the medium can be supplemented with defined or non-defined not mammal-derived compounds. An example of such a not mammal-derived compound is cod-liver oil.

Therefore in one embodiment of the current invention the medium used in the cultivation is a serum-free medium, or a serum-free medium supplemented with defined mammal-derived components, or an mammal-derived component free medium, or a protein-free medium, a protein-free medium supplemented with defined mammal-derived components, or a chemically defined medium, or a mammal-derived component free medium, or a defined protein-free medium. Examples of an mammal-derived component free medium are the CD CHO medium available from Invitrogen Corp., or the ProCHO4 available from Gibco. An example of a protein free medium is HyQ SFM4CHO available from Hyclone.

In another embodiment of the method according to the invention is the method beginning with the first transfection and ending with the recovery of the secreted heterologous immunoglobulin performed in the same medium. The term "in the same medium" denotes within the current application that beginning with the first transfection and ending with the recovery of the secreted heterologous immunoglobulin from the cultivation medium the same medium is used. This does not denote that the same additives have to be added to the medium in all steps, i.e. the medium may be supplemented with different additive in different steps of the method. Additives are compounds that are added to a medium in total to less than 20% (w/w), in one embodiment to less than 15% (w/w), in another embodiment to less than 10% (w/w). In one embodiment the medium used in the method according to the invention is the same medium in all steps and is a medium suitable for the large scale production of the secreted heterologous immunoglobulin.

It has surprisingly been found that with the method according to the invention a multiple transfected CHO cell can be obtained that has similar growth characteristics and an improved productivity compared to a one-time transfected CHO cell. The term "similar growth characteristics" denotes that the multiple transfected CHO cell grows to at least 50% of the cell density within the same time as the one-time transfected CHO cell. In another embodiment said multiple transfected CHO cell grows to at least 90% of the cell density as the one-time transfected cell. In still a further embodiment is the doubling time of the multiple transfected cell at most 150% of that of the one-time transfected cell. In one embodiment said multiple transfected CHO cell is a CHO cell transfected two or three times. In another embodiment the multiple transfected cell has an improved volumetric yield in a cultivation medium. The overall productivity of a large scale fermentation process is best determined by the volumetric yield, i.e. the amount of polypeptide per unit volume of the cultivation. This volumetric yield is the product of cell density, specific productivity of each cell and cultivation time. Thus, a cultivation with low cell density but high specific productivity will have the same volumetric yield in the same time as a cultivation with high cell density but low specific productivity in the same cultivation time. Thus, with the multiple transfected CHO cell and the method according to the invention a CHO cell is obtainable with similar growth characteristics but an improved volumetric yield/productivity compared to one-time transfected CHO cells.

The secreted heterologous immunoglobulin can be recovered from the cultivation medium with chromatographic methods known to a person of skill in the art. Therefore in one embodiment the method according to the invention comprises the final step of purifying said heterologous immunoglobulin with one or more chromatographic steps.

A vector suited for use in the method according to the invention comprises a prokaryotic origin of replication, and a first nucleic acid conferring resistance to a prokaryotic selection agent, and/or a second nucleic acid encoding the heavy chain of said heterologous immunoglobulin, and/or a third nucleic acid encoding the light chain of said heterologous immunoglobulin, and a fourth nucleic acid conferring resistance to a eukaryotic selection agent.

The comprised first nucleic acid confers resistance to the addition of a prokaryotic selection agent to the cultivation medium. Exemplary prokaryotic selection agents are e.g. ampicillin, kanamycin, chloramphenicol, tetracycline, or erythromycin. The term "a nucleic acid conferring resistance to a selection agent" and grammatical equivalents thereof denotes within the current application that the polypeptide encoded by said nucleic acid can neutralize said selection agent by modification or degradation or can counteract the effect of said selection agent. Thus, a cell comprising a nucleic acid conferring resistance to a selection agent has the ability to survive and proliferate with the selection agent present in the cultivation medium. Exemplary eukaryotic selection agents are e.g. neomycin, hygromycin, puromycin, methotrexate, Geneticin® (G418), or mycophenolic acid. The selection agent is chosen with the proviso that the prokaryotic and the eukaryotic selection agent is not a metal.

The transfection of the provided CHO cell according to the method according to the invention is performed as sequential steps of transfection and selection. CHO cells suitable in the method according to the invention are e.g. a CHO K1 cell, or a CHO DG44 cell, or a CHO XL99 cell, or a CHO DXB11 cell, or a CHO DP12 cell, or a super-CHO cell. Within the scope of the present invention, transfected cells may be obtained with substantially any kind of transfection method known in the art. For example, the nucleic acid may be introduced into the cells by means of electroporation or microinjection. Alternatively, lipofection reagents such as FuGENE 6 (Roche Diagnostics GmbH, Germany), X-tremeGENE (Roche Diagnostics GmbH, Germany), LipofectAmine (Invitrogen Corp., USA), and nucleotransfection (AMAX Corp.) may be used. Still alternatively, the nucleic acid may be introduced into the cell by appropriate viral vector systems based on retroviruses, lentiviruses, adenoviruses, or adeno-associated viruses (Singer, O., Proc. Natl. Acad. Sci. USA 101 (2004) 5313-5314).

After the transfection positive transfected cells are selected in the presence of selection agents, i.e. by selected growth. It has surprisingly been found that more than one eukaryotic selection agent can be present in the cultivation medium not interfering with growth and heterologous polypeptide expression if the cultivated CHO cell has been transfected with all required corresponding nucleic acids conferring resistance to these eukaryotic selection agents according to the current invention. It has also been found that CHO cells can be cultivated in the concomitant presence of three eukaryotic selection agents without a reduction of the doubling time to more than 150% of the doubling time of the non-transfected or one-time transfected CHO cell. Therefore, the multiple transfected CHO cell comprises nucleic acids, which are in each transfection step of the method according to the invention comprising a different, not previously transfected, nucleic acid as fourth nucleic acid which confers a new resistance not already present in said CHO cell to a different eukaryotic selection agent. Therefore, after the second transfection step a successfully transfected cell is selected for by cultivation in the concomitant presence of two different eukaryotic selection agents. After the third transfection the transfected cell can be cultivated for selection in the concomitant presence of three different eukaryotic selection agents.

Thus, the vector employed in the different transfection steps according to the method according to the invention is at

least 95% identical on the nucleic acid level except for the nucleic acid conferring resistance to a eukaryotic selection agent, i.e. the fourth nucleic acid.

For the expression of a secreted heterologous immunoglobulin the vector with which the CHO cell is transfected and which comprises a nucleic acid conferring resistance to a eukaryotic selection agent also comprises a nucleic acid encoding the light chain of said heterologous immunoglobulin and/or a nucleic acid encoding the heavy chain of said heterologous immunoglobulin. If the vector comprises only a nucleic acid encoding either the light chain of said immunoglobulin or the heavy chain of said immunoglobulin said CHO cell is also transfected in each step by another vector comprising a nucleic acid encoding the corresponding other chain of said immunoglobulin.

In one embodiment the first to fourth nucleic acid sequence comprised in the transfection vectors according to the invention (i.e. the first, second, and third transfection vector) is contained in an expression cassette. An "expression cassette" refers to a construct that contains the necessary regulatory elements, such as promoter and polyadenylation site, for expression of at least the contained nucleic acid in a cell, e.g. a promoter, a nucleic acid to be expressed, and a transcription terminator including a polyadenylation signal. The promoter contained in the expression cassette determines the amount of transcription of the operably linked nucleic acid and therewith it determines the amount of the translation of said nucleic acid. A first promoter inducing a larger amount of translation of a nucleic acid compared to a second promoter is termed a "stronger promoter" with respect to said second promoter. It is intended to produce the secreted heterologous immunoglobulin and not the polypeptide conferring resistance to a selection agent. Thus, the capacity of the host cells transcription and translation machinery has to be split up correspondingly. Therefore, in one embodiment the promoter employed for the transcription of said second and third nucleic acids is different from the promoter employed for the transcription of said fourth nucleic acid. In another embodiment is the amount of transcript of said second and third nucleic acid encoding the chains of said heterologous immunoglobulin larger than the amount of transcript of said forth nucleic acid conferring resistance to a selection agent. Thus, the promoter employed for the expression of said second and third nucleic acid is stronger than the promoter employed for the expression of said fourth nucleic acid. In another embodiment is the promoter employed for the transcription of said second and third nucleic acids the same but different from the promoter of said fourth nucleic acid. In one embodiment the promoter for the expression of said second and third nucleic acid is the CMV promoter or a variant thereof and the promoter for the expression of said fourth nucleic acid is the SV40 promoter or a variant thereof.

In a further embodiment of the method according to the invention the codon usage of said second and third nucleic acid is optimized for the expression in CHO cells. This allows a more efficient use of the transfer-RNAs present in the recombinant CHO cell. In another embodiment said second and/or third nucleic acid comprise an intronic nucleic acid sequence, in another embodiment the intronic nucleic acid is a mouse/human hybrid intron. In the genome of eukaryotic cells the genomic DNA sequences contain coding (exonic) and non-coding (intronic) nucleic acid sequences. After transcription of the DNA to the pre-mRNA, the pre-mRNA also contains these intronic and exonic nucleic acid sequences. Prior to translation the non-coding intronic nucleic acid sequences are removed during mRNA processing by splicing them out of the primary mRNA transcript to generate the mature mRNA. The splicing of the primary mRNA is controlled by a splice donor site in combination with a properly spaced apart splice acceptor site. The splice donor site is located at the 5' end and the splice acceptor site is located at the 3' end of an intronic sequence and both are only partly removed during the pre-mRNA splicing.

To produce a secreted polypeptide, the nucleic acid(s) encoding the chains of the heterologous immunoglobulin include a DNA segment that encodes a signal sequence/leader peptide. The signal sequence directs the newly synthesized polypeptide to and through the Endoplasmatic reticulum (ER) membrane where the polypeptide can be routed for secretion. The signal sequence is cleaved off by a signal peptidases during crossing of the ER membrane. As for the function of the signal sequence the recognition by the host cell's secretion machinery is essential. Therefore, the used signal sequence has to be recognized by the host cell's proteins and enzymes of the secretion machinery.

In one embodiment the method according to the invention comprises a third transfection step in step c):

(v) transfecting said CHO cell selected in (iv) with said vector comprising a fourth nucleic acid sequence different from that in the transfection vector used in (i) and (iii) conferring resistance to a third eukaryotic selection agent, which is different from said first and said second eukaryotic selection agent,

(vi) selecting a CHO cell transfected in (v) by selected growth in a cultivation medium containing said first and said second and said third eukaryotic selection agent.

In this embodiment the cultivation medium employed for the cultivation of said transfected CHO cell in step d) further comprises a third eukaryotic selection agent.

A second aspect of the current invention is a CHO cell expressing a secreted heterologous immunoglobulin obtainable with the following method:

a) providing a CHO cell, which is
 adapted to growth in suspension culture,
 adapted to growth in serum-free medium,
 mycoplasma free,

b) providing a nucleic acid comprising
 a prokaryotic origin of replication,
 a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,
 a second nucleic acid sequence encoding the heavy chain of said heterologous immunoglobulin, and a third nucleic acid sequence encoding the light chain of said heterologous immunoglobulin,
 whereby a first transfection vector is provided which comprises said provided nucleic acid and an additional fourth nucleic acid sequence conferring resistance to a first eukaryotic selection agent,
 whereby a second transfection vector is provided which comprises said provided nucleic acid and an additional fourth nucleic acid sequence different from the fourth nucleic acid in said first transfection vector conferring resistance to a second eukaryotic selection agent, whereby said second eukaryotic selection agent is different to said first eukaryotic selection agent,

c) transfecting and selecting said CHO cell, wherein said transfecting and selecting comprises the following steps in the following order:
 (i) transfecting said CHO cell with said first transfection vector,
 (ii) selecting a CHO cell transfected in (i) by selected growth in a cultivation medium containing a first eukaryotic selection agent to which the first transfection vector confers resistance,

(iii) transfecting said CHO cell selected in (ii) with said second transfection vector,
(iv) selecting a CHO cell transfected in (iii) by selected growth in a cultivation medium containing said first eukaryotic selection agent, to which the first transfection vector confers resistance, and said second eukaryotic selection agent, to which the second transfection vector confers resistance.

The term "virus free" which is used within this application denotes that the CHO cell does not contain any viral nucleic acid which would result if expressed during cultivation in harmful, in down stream processing operations not separatable products for humans.

The following examples, and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

DESCRIPTION OF THE FIGURES

FIG. **1** Annotated plasmid map of plasmid p5128.
FIG. **2** Annotated plasmid map of plasmid p5137.
FIG. **3** Annotated plasmid map of plasmid p5151.
FIG. **4** Annotated plasmid map of plasmid p5057.
FIG. **5** Annotated plasmid map of plasmid p5069.

FIG. **6** (A) Antibody titers of clones obtained after subcloning with limited dilution and of clones obtained with the method according to the invention; X-axis: (1) G24, (2) limited dilution, (3) method according to the invention; Y-axis: immunoglobulin concentration [μg/ml].

(B) Specific production rates of clones obtained after subcloning with limited dilution and of clones obtained with the method according to the invention; X-axis: (1) G24, (2) limited dilution, (3) method according to the invention; Y-axis: specific production rate [pg/d*cell].

FIG. **7** SDS-Page after protein-A HPLC purification of the antibody. For the four samples 35-45, 37-65, 39-4 and 43-16 two bands are visible, the upper being the heavy chain, the lower being the light chain. Sample 25g7 is a control antibody with antibody-related side products (above the heavy chain and between heavy and light chain). Samples: (1) Molecular weight marker, (2) 35-45, (3) 37-65, (4) 39-4, (5) 43-16), (6) 25g7, (7) Reference antibody, (8) Medium 25×.

FIG. **8** Annotated plasmid map of plasmid p6311.
FIG. **9** Annotated plasmid map of plasmid p5321.

EXAMPLES

Materials & Methods

General information regarding the nucleotide sequences of human immunoglobulins light and heavy chains is given in: Kabat, E. A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, Md. (1991). Amino acids of antibody chains are numbered according to EU numbering (Edelman, G. M., et al., Proc. Natl. Acad. Sci. USA 63 (1969) 78-85; Kabat, E. A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, Md., (1991)).

Recombinant DNA Techniques:

Standard methods were used to manipulate DNA as described in Sambrook, J., et al., Molecular cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989. The molecular biological reagents were used according to the manufacturer's instructions.

Gene Synthesis:

Desired gene segments were prepared from oligonucleotides made by chemical synthesis. The 100-600 bp long gene segments, which are flanked by singular restriction endonuclease cleavage sites, were assembled by annealing and ligation of oligonucleotides including PCR amplification and subsequently cloned into the pCR2.1-TOPO-TA cloning vector (Invitrogen Corp., USA) via A-overhangs or pPCR-Script Amp SK(+) cloning vector (Stratagene Corp., USA). The DNA sequence of the subcloned gene fragments were confirmed by DNA sequencing.

Protein Determination:

Protein concentration was determined by determining the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence.

Antibody Titer Determination:

Antibody titers were determined either by anti-human Fc ELISA or by Protein A chromatography using the autologous purified antibody as a reference.

SDS-PAGE

LDS sample buffer, fourfold concentrate (4×): 4 g glycerol, 0.682 g TRIS-Base, 0.666 g TRIS-hydrochloride, 0.8 g LDS (lithium dodecyl sulfate), 0.006 g EDTA (ethylene diamin tetra acid), 0.75 ml of a 1% by weight (w/w) solution of Serva Blue G250 in water, 0.75 ml of a 1% by weight (w/w) solution of phenol red, add water to make a total volume of 10 ml.

The culture broth containing the secreted antibody was centrifuged to remove cells and cell debris. An aliquot of the clarified supernatant was admixed with ¼ volumes (v/v) of 4xLDS sample buffer and 1/10 volume (v/v) of 0.5 M 1,4-dithiotreitol (DTT). Then the samples were incubated for 10 min. at 70° C. and protein separated by SDS-PAGE. The NuPAGE® Pre-Cast gel system (Invitrogen Corp.) was used according to the manufacturer's instruction. In particular, 10% NuPAGE® Novex® Bis-TRIS Pre-Cast gels (pH 6.4) and a NuPAGE® MOPS running buffer was used.

Western Blot

Transfer buffer: 39 mM glycine, 48 mM TRIS-hydrochloride, 0.04% by weight (w/w) SDS, and 20% by volume methanol (v/v)

After SDS-PAGE the separated antibody chains were transferred electrophoretically to a nitrocellulose filter membrane (pore size: 0.45 μm) according to the "Semidry-Blotting-Method" of Burnette (Burnette, W. N., Anal. Biochem. 112 (1981) 195-203).

Example 1

Expression Vector for Expressing an Anti-Aβ Antibody

An example (preferably monoclonal) antibody for which a cell line for expression can be obtained according to the current invention is an antibody against the amyloid β-A4 peptide (anti-Aβ antibody). Such an antibody and the corresponding nucleic acid sequences are, for example, reported in WO 2003/070760 or US 2005/0169925 or in SEQ ID NO: 1 to 12.

The anti-Aβ antibody expressing Chinese hamster ovary (CHO) cell line was generated by three successive complete transfections and selection campaigns.

A genomic human κ-light chain constant region gene segment (C-kappa, C_L) was added to the light chain variable region of the anti-Aβ antibody, while a human γ1-heavy chain constant region gene segment (C_{H1}-Hinge-C_{H2}-C_{H3}) was added to the heavy chain variable region of the anti-Aβ antibody. The complete κ-light and γ1-heavy chain antibody genes were then joined with a human cytomegalovirus

(HCMV) promoter at the 5'-end and a human immunoglobulin polyadenylation signal sequence at the 3'-end.

a) Heavy Chain Expression Cassette

The transcription unit of the anti-Aβ antibody heavy chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus,
- a 5'-untranslated region derived from a human antibody germline gene,
- the anti-Aβ antibody heavy chain variable domain including a signal sequence derived from a human antibody germline gene,
- a human/mouse heavy chain hybrid intron 2 including the mouse Ig heavy chain enhancer element (see e.g. (Neuberger, M. S., EMBO J. 2 (1983) 1373-1378),
- the genomic human γ1-heavy chain gene constant region,
- the human immunoglobulin γ1-heavy chain polyadenylation ("poly A") signal sequence,
- the unique restriction sites AscI and SgrAI at the 5'- and 3'-end, respectively.

b) Light Chain Expression Cassette

The transcription unit of the anti-Aβ antibody light chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (HCMV),
- a 5'-untranslated region derived from a human antibody germline gene,
- the anti-Aβ antibody light chain variable region including a signal sequence derived from a human antibody germline gene,
- a human/mouse κ-light gene hybrid intron 2 including the mouse Ig χ-light chain enhancer element (Picard and Schaffner, A lymphocyte-specific enhancer in the mouse immunoglobulin kappa gene. Nature 307 (1984) 80-82),
- the human κ-light gene constant region (C-kappa),
- the human immunoglobulin κ-polyadenylation ("poly A") signal sequence,
- the unique restriction sites Sse8387 and FseI at the 5'- and 3'-end, respectively.

c) Expression Plasmids 5128, 5137, and 5151

For expression and production of the anti-Aβ antibody the light and heavy chain expression cassettes were placed on a single expression vector (heavy chain upstream of light chain in clockwise orientation). Three identical expression vectors were generated differing only in the selectable marker gene included, in particular, in the gene conferring resistance to the selection agent neomycin, hygromycin, or puromycin. The vectors also include a mouse DHFR gene which was not used for selection or amplification.

The expression vectors contain beside the light and heavy chain expression cassette the following elements:

- a selectable marker (either a neomycin, hygromycin or puromycin resistance gene),
- an origin of replication allowing for the replication of the plasmid in *E. coli,*
- a beta-lactamase gene which confers ampicillin resistance in *E. coli,*
- a mouse derived DHFR gene.

The plasmid map of the expression vector 5128 containing a hygromycin selectable marker gene is shown in FIG. **1**. The plasmid map of the expression vector 5137 containing a neomycin selectable marker gene is shown in FIG. **2**. The plasmid map of the expression vector 5151 containing a puromycin selectable marker gene is shown in FIG. **3**.

Example 2

Transfection and Selection of a CHO Cell Expressing an Anti-Aβ Antibody

Parent CHO-K1 cells, pre-adapted to growth in serum-free suspension culture in synthetic animal component free ProCHO4 medium (Cambrex Corp.) containing 8 mM glutamine and 1×HT supplement (Gibco/Invitrogen) were used as host cell line. This supplemented ProCHO4 medium is designated in the following as ProCHO4-complete medium. The adherent growing CHO-K1 parent cell line was received from ATTC as ATCC CCL-61.

The preadapted parent host cells were propagated in suspension in synthetic, animal component-free ProCHO4-complete medium under standard humidified conditions (95%, 37° C., and 5% CO_2). On regular intervals depending on the cell density the cells were splitted into fresh medium. The cells were harvested by centrifugation in the exponential growth phase, washed once in sterile Phosphate Buffered Saline (PBS) and resuspended in sterile PBS.

Prior to transfection the anti-Aβ antibody expressing plasmids were linearized within the β-lactamase gene (*E. coli* ampicillin resistance marker gene) using the restriction endonuclease enzyme PvuI or AviII. The cleaved DNA was precipitated with ethanol, dried under vacuum, and dissolved in sterile PBS.

In general, for transfection, the (parent or already transfected) CHO cells were electroporated with 20-50 μg linearized plasmid DNA per approximately 10^7 cells in PBS at room temperature. The electroporations were performed with a Gene Pulser XCell electroporation device (Bio-Rad Laboratories) in a 2 mm gap cuvette, using a square wave protocol with a single 180 V pulse. After transfection, the cells were plated out in ProCHO4-complete medium in 96-well culture plates. After 24 h of growth a solution containing one or more selection agents were added (ProCHO4-complete selection medium; G418: 400 μg/ml; hygromycin: 600 μg/ml; puromycin: 8 μg/ml). Once a week the ProCHO4-complete selection medium was replaced. The antibody concentration of the anti-Aβ antibody was analyzed with an ELISA assay specific for human IgG1 in the culture supernatants.

For selection of high-yield anti-Aβ antibody production cell lines the productivity was tested in ProCHO4-complete selection medium after propagation in 6-well culture plates, T-flasks and/or Erlenmeyer shake flasks using an anti-human IgG1 ELISA and/or analytic Protein A HPLC.

Subclones were obtained by two methods, Limiting Dilution (LD) and Fluorescence Activated Cell Sorting (FACS).

Limiting Dilution:

For limiting dilution cells were plated out in ProCHO4-conditioned medium (consisting of 50% (v/v) fresh ProCHO4-complete selection medium and 50% (v/v) ProCHO4-complete conditioned selection medium derived from the cells to be propagated) at a cell density of 0.5-2 cells per 0.1 ml medium per well of a 96-well culture plate. Once a week the medium was replaced by ProCHO4-complete selection medium. The antibody concentration of the anti-Aβ antibody was analyzed by an ELISA assay specific for human IgG1 in the culture supernatants.

Single Cell Deposition by Flow Cytometry Including Identification and Isolation of Clones:

The identification and isolation of stably transfected clones was performed with the aid of a cell surface labeling technique using fluorescently tagged Protein A that binds to secreted but still membrane-attached antibodies. The fluorescence intensity of the stained cells was used as criterion for cell selection.

In the case of fluorescence activated cell sorting the electroporated population of cells were directly seeded into T-flasks in ProCHO4-complete medium. The appropriate selection agent or agents (G418, hygromycin, and/or puromycin) was/were added to the culture one day after transfection and the transfectant pool was expanded.

Cells from the expanded transfectant pool were first treated with Accumax (PAA Laboratories) for 15 minutes at 37° C. and then passed through a 40 μM nylon mesh to remove remaining large cell aggregates. The cells were collected by centrifugation, resuspended in PBS containing 5% FCS (Gibco/Invitrogen) at a cell density of 10^6 to 10^7 cells/ml and incubated for 20 minutes on ice. Thereafter, the cells were stained with 10 ng/ml Protein A Alexa Fluor 488 (Molecular Probes Inc.) in a volume of 8 ml FCS-PBS for 30 minutes on ice in the dark. Afterwards, the cells were washed once with 5% FCS-PBS and once with ProCHO4 medium containing 8 mM Ultra Glutamine (Cambrex Corp.), 1×HT supplement and 5% FCS. Finally the cells were resuspended in the supplemented ProCHO medium used for washing at a cell density of 10^6 to 10^7 cells/ml and transferred to a BD FACSAria cell sorter (BD Biosciences).

Single cells were sorted by flow cytometry and deposited in wells of 96-well culture plates containing of ProCHO4-conditioned medium. The selected and deposited cells encompassed cells with the top 10%, 7%, or 4% of fluorescence intensity of the gated live cells. After 48 hours ProCHO4 complete selection medium containing the appropriate selection agent in 2-fold concentration was added to each well. Once a week the medium was replaced with ProCHO4-complete selection medium. The antibody concentration of the anti-Aβ antibody was analyzed with an ELISA assay specific for human IgG1 in the culture supernatants.

Transfection and Selection Steps:

For the first transfection and selection step the plasmid 5137 has been used. Plasmid 5137 has been transfected with electroporation into parent cell line adapted to growth in ProCHO4-complete medium. The transfected cells were cultivated in ProCHO4-complete medium supplemented with up to 700 μg/ml G418 in 96 well plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. Approximately 1000 clones have been tested and the selected of them were further cultivated in 24-well plates, 6-well plates and subsequently in shaker flasks. The growth and productivity of approximately 20 clones was assessed in static and suspension cultures by anti-human IgG1 ELISA and/or analytic protein A HPLC. The best clone (best clone does not denote the most productive clone it denotes the clone with the best properties for the further steps) was subcloned by limited dilution in ProCHO4-conditioned medium supplemented with 700 μg/ml G418. The selected clone was named 8C8.

For the second transfection and selection step the plasmid 5128 has been used. Plasmid 5128 has been transfected with electroporation into cell line clone 8C8 cultivated in ProCHO4-complete medium supplemented with 700 μg/ml G418. The transfected cells were expanded for about two to three weeks in ProCHO4-conditioned medium supplemented with 200 μg/ml G418 and 300 μg/ml hygromycin (ProCHO4-double selection medium). Single antibody secreting cells were identified and deposited on the basis of their fluorescence intensity after staining with a Protein A Alexa Fluor conjugate by FACS analysis. The deposited cells were cultivated in ProCHO4-double selection medium in 96 well plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. Approximately 500 clones have been tested and the selected of them were further cultivated in 24-well plates, 6-well plates and subsequently in shaker flasks. The growth and productivity of approximately 14 clones was assessed in static and suspension cultures by anti-human IgG1 ELISA and/or analytic Protein A HPLC. The selected clone was named 4F5.

For the third transfection and selection step the plasmid 5151 has been used. Plasmid 5151 has been transfected with electroporation into cell line clone 4F5 cultivated in ProCHO4-double selection medium. The transfected cells were expanded for about two to three weeks in ProCHO4-triple selection medium (ProCHO4-conditioned medium supplemented with 200 μg/ml G418 and 300 μg/ml hygromycin and 4 μg/ml puromycin). Single antibody secreting cells were identified and deposited on the basis of their fluorescence intensity after staining with a Protein A Alexa Fluor conjugate by FACS analysis. The deposited cells were cultivated in ProCHO4-triple selection medium in 96 well plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. Approximately 500 clones have been tested and the selected of them were further cultivated in 24-well plates, 6-well plates and subsequently in shaker flasks. The growth and productivity of approximately 10 clones was assessed in static and suspension cultures by anti-human IgG1 ELISA and/or analytic protein A HPLC. The selected clone was named 20F2.

Clone 20F2 has been selected based on his growth, productivity, and product quality characteristics after growth in fed-batch suspension culture in ProCHO4-triple selection medium, i.e. in the concomitant presence of the three selecting agents G418, hygromycin, and puromycin.

Clone Characteristics:

As can be seen from the following table the doubling time and cell density after three days of cultivation were comparable when the basic cell line CHO-K1 (wild-type) and the selected clones are compared.

TABLE 1

Growth characteristics				
Clone	Doubling time [h]	Starting cell density [10^6 cells/ml]	Cell density at day 3 [10^6 cells/ml]	Viability at day 3 [%]
CHO-K1 (wild-type)	22-23	3	18-20	97-98
8C8	26-28	3	12-15	96-98
4F5	22-24	3	24-27	96-97
20F2	24-26	2	23-26	97-98

Example 3

Stability of Clone 20F2 Expressing an Anti-Aβ Antibody

Stability of growth and product formation was evaluated in sequential cell subculture over a time period of 60 days (about 60 generations) in the presence and absence of the selection agents (with and without antibiotics). The cultivation was performed as described above.

TABLE 2

Characteristics of clone 20F2.

Parameter	Clone 20F2 cultivation in the presence of three selection agents	Clone 20F2 cultivation in the absence of selection agents
Mean value viability [%]	97	97
Mean value doubling time [h]	27	26
Mean value SPR [pg/c/d]	11	9

Following extensive passage (up to generation 60) no evidence was obtained indicating that the anti-Aβ antibody producing clone 20F2 was unstable with respect to cell growth and product formation in the presence or absence of the three selection agents, respectively.

Example 4

Expression Vector for Expressing an Anti-P-Selectin Antibody

Another example (preferably monoclonal) antibody for which a cell line for expression can be obtained according to the current invention is an antibody against the human P-Selectin glycoprotein (anti-P-Selectin antibody). Such an antibody and the corresponding nucleic acid sequences are for example described in WO 2005/100402, or US 2005/0226876 or SEQ ID NO: 13 to 18.

The anti-P-Selectin antibody expressing Chinese hamster ovary cell line was generated by two successive complete transfections and clone selection campaigns.

A genomic human kappa-light chain constant region gene segment (C-kappa) was added to the light chain variable region of the anti-P-Selectin antibody, whereas a human gamma 4-heavy chain constant region gene segment (C_{H1}-Hinge-C_{H2}-C_{H3}) was added to the heavy chain variable region of the anti-P-Selectin antibody. The complete kappa-light and gamma 4-heavy chain antibody genes were then joined with a human cytomegalovirus immediate early promoter and enhancer (CMV IE) at the 5'-end and the Simian Virus 40 early polyadenylation (SV 40 early poly A) signal sequence at the 3'-end.

a) Heavy Chain Expression Cassette

The transcription unit of the anti-P-Selectin antibody heavy chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (CMV IE),
- a 5'-untranslated region (5' UTR),
- the coding sequence for the anti-P-Selectin antibody gamma 4-heavy chain including a signal peptide in an intron-exon gene structure,
- the SV 40 early poly A signal sequence.

b) Light Chain Expression Cassette

The transcription unit of the anti-P-Selectin antibody light chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (CMV IE),
- a 5'-untranslated region (5' UTR),
- the coding sequence for the anti-P-Selectin kappa-light chain in an intron-exon gene structure,
- the SV 40 early poly A signal sequence.

c) Expression Plasmids 5057 and 5069

For the expression and production of the anti-P-Selectin antibody the light and heavy chain expression cassettes were placed on a single expression vector (light chain upstream of heavy chain). Two identical expression vectors were generated differing only in the selectable marker gene included, in particular, the murine dihydrofolate reductase (DHFR) gene or a neomycin resistance gene.

The expression vectors contain beside the light and heavy chain expression cassette the following elements:

- a selectable marker, either the murine DHFR gene or a gene conferring resistance to the selection agent neomycin under the control of the SV40 early promoter and origin,
- an origin of replication allowing for the replication of the plasmid in *E. coli* taken from pUC19 (pUC origin),
- a beta-lactamase gene which confers ampicillin resistance in *E. coli*.

The plasmid map of the expression vector 5057 containing the murine DHFR marker gene is shown in FIG. **4**. The plasmid map of the expression vector 5069 containing a neomycin selectable marker gene is shown in FIG. **5**.

Example 5

Transfection and Selection of a CHO Cell Line Expressing an Anti-P-Selectin Antibody

CHO-K1 cells, pre-adapted to growth in serum-free suspension culture in protein-free HyQ SFM4CHO medium (Hyclone, Cat. No. SH30549) supplemented with defined animal-derived components (cholesterol from ovine wool and cod-liver oil) were used as the host cell line. The cells were propagated in shake flasks in protein-free HyQ SFM4CHO medium under standard humidified conditions (95%, 37° C., and 5% CO_2) and under constant agitation at 150 rpm/min. Depending on the cell density the cells were split into fresh medium.

The adherent CHO-K1 cell lines had been obtained from the American Type Culture Collection as ATCC CCL-61.

First Transfection and Selection

Prior to transfection the expression plasmid 5057 was linearized within the beta-lactamase gene using the restriction enzyme PvuI. The cleaved DNA was purified using QiaQuick spin columns (Qiagen) according to the manufacturer's recommendations.

Transfection was carried out by electroporation using Gene Pulser XCell (BIO-RAD) and 0.2 cm-cuvettes (BIO-RAD, Cat. No. 165-2086). For transfection 10^6 to 10^7 CHO-K1 cells were harvested by centrifugation, resuspended in PBS, transferred to the cuvette and mixed with 20-50 µg linearized plasmid DNA. The cells were exposed to a single square wave pulse (160 V, 15 ms) and subsequently diluted in HyQ SFM4CHO medium to a density of approx. 4×10^5 cells/ml and seeded in a T75 cell culture flask. After 48 hours of propagation without the supplementation of a selection agent, the cells were diluted in HyQ SFM4CHO medium supplemented with 200 nM MTX to a density of 10^4 to 10^5 cells/ml and seeded in 96-well plates with 3-7000 cells per well. After approx. two weeks, fresh medium was added per well and after additional two weeks the culture medium was completely replaced by fresh medium. Four days later the culture supernatants were tested for antibody production by anti-human Fc ELISA. In total approximately 600 clones were screened.

45 clones with antibody titers of more than 10 µg/ml were picked and transferred to 48-well plates. The clones were expanded to shaker flasks over additional passages and subsequently transferred to serum free production medium for the final productivity assessment. A 125 ml shaker flask was inoculated with 10^5 to 10^6 cells/ml in medium supplemented with 200 nM MTX. Viable cell density and viability were monitored over one week. Antibody titers were measured by Protein A chromatography on the final day. Based on these

data, clone G24 was selected for further development. G24 reached a maximal viable cell density of 3.3×10^6 cells/ml. The antibody titer was 402 μg/ml. The average specific production rate (SPR) was 28 pg/(cell*d).

Second Transfection and Selection:

Clone G24 was subjected to a second transfection. For the second transfection plasmid 5069 was used. Linearization and purification of the plasmid as well as electroporation of G24 were performed as described for the first transfection. After 48 hours of propagation without selection pressure, the cells were diluted in HyQ SFM4CHO medium supplemented with 200 nM MTX and 400 μg/ml G418 to a density of 10^3 to 10^4 cells/ml and seeded in 96-well plates with 500 cells per well. After approx. two weeks, fresh medium was added per well and after an additional week the culture medium was completely replaced by fresh medium. Four days later the culture supernatants were tested for antibody production by anti-human Fc ELISA. In total approximately 220 clones were screened.

Then 13 clones with antibody titers of more than 150 μg/ml were picked and transferred to 24-well plates. The clones were expanded to shaker flasks over additional passages and subsequently transferred to serum free production medium for the final productivity assessment. A shaker flask was inoculated with 10^5 to 10^6 cells/ml in 50 ml medium supplemented with 200 nM MTX and 400 μg/ml G418. Viable cell density and viability were monitored over one week. Antibody titers were measured by Protein A chromatography on the final day. Based on these data, clone G24_x6 was considered the best clone. G24_x6 reached a maximal viable cell density of 3.0×10^6 cells/ml. The antibody titer was 685 μg/ml. The average specific production rate (SPR) from was 48 pg/(cell*d).

Limiting Dilution:

To compare the method according to the invention with simple subcloning with respect to their effect on productivity we subjected clone G24 to limited dilution or single cell deposition in 96-well plates.

For limiting dilution the cells were seeded in 96-well plates in HyQ SFM4CHO medium supplemented with 50% (v/v) conditioned medium, 10% FCS and 200 nM MTX at 0.5 cells/well. Alternatively 1 cell/well was deposited in 96-well plates by FACS. After 10 days, fresh HyQ SFM4CHO medium, 200 nM MTX without FCS was added per well and after an additional week the culture medium was completely replaced by HyQ SFM4CHO medium, 200 nM MTX. Four days later the culture supernatants were tested for antibody production by anti-human Fc ELISA. In total approximately 230 clones were screened.

Eleven subclones with antibody titers of more than 130 μg/ml were transferred to 24-well plates. After passages in 6-well plates, the clones were transferred to shaker flasks and subsequently transferred to serum free production medium for the final productivity assessment. A shaker flask was inoculated with 10^5 to 10^6 cells/ml in medium supplemented with 200 nM MTX. Viable cell density and viability were monitored over one week. Antibody titers were measured by Protein A chromatography on the final day. Based on these data G24__13 was considered the best clone. G24__13 reached a maximal viable cell density of 3.6×10^6 cells/ml. The antibody titer was 472 μg/ml. The average the specific production rate (SPR) was 31 pg/(cell*d).

Table 3 summarizes the productivity data of best performing subclone G24__13 and the best performing clone G24_x6 obtained with the method according to the invention in comparison to their parental clone G24. With the method according to the invention a clone with volumetric and specific productivity increased by more than 50% can be obtained whereas after subcloning only a minor increase of both parameters was observed.

FIG. **6** shows an overview of the volumetric (A) and specific (B) productivities of all subclones of G24 that had been investigated in shake flasks. As can be seen, the average volumetric and specific productivity of the clones obtained with the method according to the invention was significantly higher than after subcloning.

TABLE 3

Productivity of the best producing clones compared to the parental clone G24.

	G24	G24__13 (Subclone)	G24__x6 (method according to the invention)
Antibody concentration in the supernatant [μg/ml]	402	472	685
SPR pg/(cell*d)]	28	31	48
Max. cell density [10^5/ml]	33	36	30

Clone Characteristics:

As can be seen from the following table the doubling time and the cell density after three days of cultivation were comparable when the one-time transfected cell line G24 and the selected clones are compared.

TABLE 4

Growth characteristics

Clone	Doubling time [h]	Starting cell density [10^6 cells/ml]	Cell density at day 3 [10^6 cells/ml]	Viability at day 3 [%]
G24	29	0.3	0.7	91
G24__13	27	0.3	2.0	91
G24__x6	24	0.3	2.5	93

Example 6

Transfection and Selection of a CHO Cell Line Expressing an Anti-P-Selectin Antibody

CHO-DG44 cells pre-adapted to growth in serum-free suspension culture in protein-free HyQ SFM4CHO medium (Hyclone, Cat. No. SH30549) were used as the host cell line. The host cell line was cultured in commercial medium HyQ SFM4CHO-utility (Hyclone, Cat. No. SH30516) during transfections, screening and subcloning steps.

First Transfection and Selection

Prior to transfection the expression plasmid 5057 (FIG. **4**) was linearized within the beta-lactamase gene using the restriction enzyme PvuI.

The transfection of the host cell line was performed by nucleotransfection provided by AMAXA (Nucleofector Kit T, Cat. No. VCA-1002, Transfection program U-17). Cells were cultured in medium supplemented with 10% fetal calf serum for 48 h after transfection.

Transfected cells were plated on 96-well plates with 1000 cells per well in medium supplemented with 10% fetal calf serum in the presence of 40 nM methotrexate (MTX) as selection agent and incubated for approx. three weeks.

Antibody concentration was determined by ELISA in the supernatant of the 96-well plates. About 400 primary clones were screened. Twenty-four clones with the highest antibody

productivity were transferred to 24-well plates and cultivated in the presence of the selection agent without supplementation with fetal calf serum. Product quality was analyzed by Western Blotting detecting light and heavy antibody chains. Nine clones which showed the highest productivity and which expressed antibody without detectable antibody derived side products (Western blot) were expanded into shake flasks.

Productivity was analyzed in batch shake flasks after 7 and 10 days of incubation. Product quality was assessed by SDS-PAGE after Protein-A HPLC purification (FIG. **7**). Best product concentration was reached with clone 43-16. Best specific productivity per cell was achieved with clone 35-45. Both clones showed no detectable side products in the SDS-PAGE. Both clones were selected for subcloning by limiting dilution.

Parental clones 35-45 and 43-16 were subcloned by limiting dilution on 96-well plates in commercial HyQ medium supplemented with 5% (v/v) fetal calf serum in the presence of 20 nM MTX. After 20 days of incubation antibody production was screened by ELISA. Best subclones in terms of productivity were expanded to shake flasks and subsequently transferred to serum free production medium for the final productivity assessment. The two best subclones, 35-45-F2 and 43-16-A10, of the parental clones 35-45 and 43-16 were assessed in standard batch shake flask assay. Productivity was 270 μg/ml and 185 μg/ml after 7 days and 337 μg/ml and 343 μg/ml after 10 days, respectively.

Second Transfection and Selection:

Subclone 43-16-A10 was transfected with the expression vector p5069 (FIG. **5**) using the nucleofection method (Amaxa Nucleofector Kit T, VCA-1002, Transfection program U-17). The second transfection was also carried out in Hyclone medium: HyQ SFM4CHO-utility (Cat. No. SH30516) supplemented with 10% fetal calf serum and 20 nM MTX. Two days after the second transfection cells were transferred to 96-well plates with 1000 cells per well. As second selection agent 250 μg/ml G418 was added.

After cultivation for two weeks more than 2000 primary wells were screened by antibody titer determination by anti-human Fc ELISA. Fifty clones with highest productivity were transferred into 24-well plates and screened a second time by anti-human Fc ELISA three days later. All clones were transferred to 6-well plates and screened by anti-human Fc ELISA three days later. The six clones with the best productivity were directly subcloned from the 6-well plate stage.

Limiting Dilution:

The best parental clones of the second transfection and selection round 43-16A10_S1, 43-16A10_S13, 43-16A10_S14, 43-16A10_S19, 43-16A10_S24, 43-16A10_S43 were subcloned by limiting dilution. The product quality of the twelve best subclones was assessed in SDS-PAGE and Western-Blotting from the 24-well stage. No unwanted antibody related side products were detected.

Three subclones, 43-16-A10-S1-16, 43-16-A10-S24-11, and 43-16-A10-S43-14, were selected according to their productivity in 6-well plates for the expansion in shake flasks. They were transferred to serum free production medium for the final productivity assessment. Their productivity was compared to the subclone after the first transfection, clone 43-16-A10. The productivity was increased twofold for two of the clones after the second transfection and selection, 43-16-A10-S1-16 and 43-16-A10-S24-11, from 221 μg/ml after 7 days in the batch shake flask to 436 μg/ml and 407 μg/ml, respectively. After 10 days incubation in the batch shake flask the productivity increased from 306 μg/ml to 683 μg/ml and 446 μg/ml, respectively.

The specific productivity per cell increased as well from 17 pg/cell/day for the clone 43-16-A10 after the first transfection to 40 pg/cell/day for the first transfected clone 43-16-A10-S1-16 and to 33 pg/cell/day for the second transfected clone 43-16-A10-S24-11. The doubling time was not affected by the second transfection. The doubling time for the clone 43-16-A10 after the first transfection was 33 h and it was 32 h for both clones 43-16-A10-S1-16 and 43-16-A10-S24-11.

Example 7

Expression Vector for Expressing an Anti-IL-13Rα Antibody

Another example (preferably monoclonal) antibody for which a cell line for expression can be obtained according to the current invention is an antibody binding to the IL-13 Receptor alpha 1 (anti-IL-13Rα1 anti-IL-13Rα antibody). Such an antibody and the corresponding nucleic acid sequences are for example described in WO 2006/072564 or SEQ ID NO: 19 to 28.

A genomic human kappa-light chain constant region gene segment (C-kappa) was added to the light chain variable region of the anti-IL-13Rα antibody whereas a human gamma 1-heavy chain constant region gene segment (C_{H1}-Hinge-C_{H2}-C_{H3}) was added to the heavy chain variable region of the anti-IL-13Rα antibody. The expression plasmid 5321 comprises an expression cassette for the anti-IL-13Rα antibody γ1-heavy chain, and the anti-IL-13Rα antibody κ-light chain, and a nucleic acid encoding the murine DHFR gene. An annotated plasmid map is shown in FIG. **9**.

a) Heavy Chain Expression Cassette

The transcription unit of the anti-IL-13Rα antibody conjugate heavy chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (CMV IE),
- a 5'-untranslated region (5' UTR),
- the coding sequence for the anti-IL-13Rα antibody gamma 1-heavy chain conjugate including a signal peptide in an intron-exon gene structure,
- the human gamma 1-immunoglobulin polyadenylation signal sequence.

b) Light Chain Expression Cassette

The transcription unit of the anti-IL-13Rα antibody light chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (CMV IE),
- a 5'-untranslated region (5' UTR),
- the coding sequence for the anti-IL-13Rα kappa-light chain in an intron-exon gene structure,
- the human immunoglobulin kappa-polyadenylation signal sequence.

c) Expression Plasmids

For the expression and production of the anti-IL-13Rα antibody conjugate the light and heavy chain expression cassettes were placed on a single expression vector (light chain upstream of heavy chain). Two identical expression vectors were generated differing only in the selectable marker gene included, in particular, the murine DHFR gene and both the murine DHFR gene and a hygromycin resistance gene.

The expression vectors contain beside the light and heavy chain expression cassette the following elements:

- an origin of replication allowing for the replication of the plasmid in *E. coli* (pUC origin),
- a beta-lactamase gene which confers ampicillin resistance in *E. coli*.

Example 8

Transfection and Selection of a CHO Cell Line Expressing an Anti-IL-13Rα Antibody

For the first transfection and selection step the plasmid 5321 has been used. Plasmid 5321 has been transfected with electroporation into parent cell line adapted to growth in ProCHO4-complete medium. The transfected cells were cultivated in HyQSFMCHO-medium (HyClone) supplemented with up to 200 nM methotrexate in plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. The clones have been tested and the selected of them were further cultivated in 24-well plates, 6-well plates and subsequently in shaker flasks. The growth and productivity was assessed in static and suspension cultures by anti-human IgG1 ELISA and/or analytic Protein A HPLC. The best clone (best clone does not denote the most productive clone it denotes the clone with the best properties for the further steps) was selected. The selected clone was named 200_019. Productivity was 90 μg/ml with an average specific production rate of 7 pg/cell*d after 7 days of cultivation.

For the second transfection and selection step a plasmid with a DHFR and hygromycin resistance gene has been used. The plasmid has been transfected with electroporation into the selected cell line cultivated in HyQSFMCHO-medium (HyClone) supplemented with up to 200 nM methotrexate. The double selection medium contained in addition 300 μg/ml hygromycine B. Single antibody secreting cells were identified and deposited on the basis of their fluorescence intensity after staining with a Protein A Alexa Fluor conjugate by FACS analysis. The selected clone was named 5_17_35. Productivity was 150 μg/ml with an average specific production rate of 10 pg/cell*d after 7 days of cultivation.

Example 9

Expression Vector for Expressing an Anti-CD4 Antibody Conjugate

Another example (monoclonal) antibody for which a cell line for expression can be obtained according to the current invention is an antibody against the human CD4 surface receptor (anti-CD4 antibody) which is conjugated to two to eight antifusogenic peptides. Such an antibody and the corresponding nucleic acid sequences are for example reported in PCT/EP2008/005894 or SEQ ID NO: 29 to 40.

A genomic human kappa-light chain constant region gene segment (C-kappa) was added to the light chain variable region of the anti-CD4 antibody of SEQ ID NO: 39, whereas a human gamma 1-heavy chain constant region gene segment (C_{H1}-Hinge-C_{H2}-C_{H3}) was added to the heavy chain variable region of the anti-CD4 antibody of SEQ ID NO: 36. The expression plasmid 6311 comprises an anti-CD4 antibody γ1-heavy chain, which is joint at the last but one C-terminal amino acid, i.e. the C-terminal lysine residue of the heavy chain is removed, with a nucleic acid encoding an antifusogenic peptide of SEQ ID NO: 41 via the peptidic glycine-serine linker of SEQ ID NO: 42, and a anti-CD4 antibody κ-light chain, and a nucleic acid conferring resistance to the selectable marker neomycin. An annotated plasmid map is shown in FIG. **8**.

a) Heavy Chain Expression Cassette

The transcription unit of the anti-CD4 antibody conjugate heavy chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (CMV IE),
- a 5'-untranslated region (5' UTR),
- the coding sequence for the anti-CD4 antibody gamma 1-heavy chain conjugate including a signal peptide in an intron-exon gene structure,
- the SV 40 early poly A signal sequence.

b) Light Chain Expression Cassette

The transcription unit of the anti-CD4 antibody conjugate light chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (CMV IE),
- a 5'-untranslated region (5' UTR),
- the coding sequence for the anti-CD4 kappa-light chain in an intron-exon gene structure,
- the SV 40 early poly A signal sequence.

c) Expression Plasmids

For the expression and production of the anti-CD4 antibody conjugate the light and heavy chain expression cassettes were placed on a single expression vector (light chain upstream of heavy chain). Three identical expression vectors were generated differing only in the selectable marker gene included, in particular, a neomycin resistance gene, a puromycin resistance gene, and a hygromycin resistance gene.

The expression vectors contain beside the light and heavy chain expression cassette the following elements:

- an origin of replication allowing for the replication of the plasmid in *E. coli* taken from pUC18 (pUC origin),
- a beta-lactamase gene which confers ampicillin resistance in *E. coli*.

Example 10

Transfection and Selection of a CHO Cell Line Expressing an Anti-CD4 Antibody Conjugate

Transfection and Selection Steps:

For the first transfection and selection step the plasmid 6311 has been used. Plasmid 6311 has been transfected with electroporation into parent cell line adapted to growth in ProCHO4-complete medium. The transfected cells were cultivated in ProCHO4-complete medium supplemented with up to 700 μg/ml G418 in 96 well plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. Approximately 5000 clones have been tested and the selected of them were further cultivated in 24-well plates, 6-well plates and subsequently in shaker flasks. The growth and productivity of approximately 15 clones was assessed in static and suspension cultures by anti-human IgG1 ELISA and/or analytic Protein A HPLC. The best clone (best clone does not denote the most productive clone it denotes the clone with the best properties for the further steps) was subcloned by limited dilution in ProCHO4-conditioned medium supplemented with 700 μg/ml G418.

Subclones were obtained by two methods, Limiting Dilution (LD) and Fluorescence Activated Cell Sorting (FACS).

Limiting Dilution:

For limiting dilution cells were plated out in ProCHO4-selection medium at a cell density of 0.5-2 cells per 0.1 ml medium per well of a 96-well culture plate.

Single Cell Deposition by Flow Cytometry Including Identification and Isolation of Clones:

In the case of fluorescence activated cell sorting the electroporated population of cells were directly seeded into T-flasks in ProCHO4-complete medium. The appropriate selection agent or agents (G418, hygromycin, and/or puromycin) was/were added to the culture one day after transfection and the transfectant pool was expanded. The growth and productivity of approximately 112 clones was assessed in

static and suspension cultures by anti-human IgG1 ELISA and/or analytic Protein A HPLC. The selected clone was named I-17.

For the second transfection and selection step a plasmid with a hygromycin resistance gene has been used. The plasmid has been transfected with electroporation into cell line clone I-17 cultivated in ProCHO4-complete medium supplemented with 700 μg/ml G418. The transfected cells were expanded for about two to three weeks in ProCHO4-conditioned medium supplemented with 200 μg/ml G418 and 300 μg/ml hygromycin (ProCHO4-double selection medium). Single antibody secreting cells were identified and deposited on the basis of their fluorescence intensity after staining with a Protein A Alexa Fluor conjugate by FACS analysis. The deposited cells were cultivated in ProCHO4-double selection medium in 96 well plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. The selected clone was named 24_16. For the third transfection and selection step a plasmid with a puromycin resistance gene has been used. The plasmid has been transfected with electroporation into cell line clone 24_16 cultivated in ProCHO4-double selection medium. The transfected cells were expanded for about two to three weeks in ProCHO4-triple selection medium (ProCHO4-conditioned medium supplemented with 200 μg/ml G418 and 300 μg/ml hygromycin and 4 μg/ml puromycin). Single antibody secreting cells were identified and deposited on the basis of their fluorescence intensity after staining with a Protein A Alexa Fluor conjugate by FACS analysis. The deposited cells were cultivated in ProCHO4-triple selection medium in 96 well plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. The selected clone was named 1_24.

Clone Characteristics:

As can be seen from the following table the doubling time and the cell density after three days of cultivation were comparable when the basic cell line CHO-K1 (wild-type) and the selected clones are compared.

TABLE 5

Growth characteristics

Clone	Doubling time [h]	Starting cell density [10^6 cells/ml]	Cell density at day 3 [10^6 cells/ml]	Viability at day 3 [%]
CHO-K1 (pre adapted)	22-25	3	18-22	96-98
I-17	25-30	3	13-15	95-97
24_16	25-30	3	15-16	95-96
1_24	30-32	3	12-14	95-97

The invention claimed is:

1. A method for the recombinant production of a heterologous immunoglobulin in a CHO cell which is secreted to the cultivation medium comprising:

a) providing a CHO cell, wherein said CHO cell is
adapted to growth in suspension culture,
adapted to growth in serum-free medium, and
mycoplasma free,

b) providing a nucleic acid comprising
i) a prokaryotic origin of replication,
ii) a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,
iii) a second nucleic acid sequence encoding the heavy chain of said heterologous immunoglobulin, and a third nucleic acid sequence encoding the light chain of said heterologous immunoglobulin,

whereby a first transfection vector is provided which comprises said provided nucleic acid and an additional fourth nucleic acid sequence conferring resistance to a first eukaryotic selection agent,

whereby a second transfection vector is provided which comprises said provided nucleic acid and an additional fourth nucleic acid sequence conferring resistance to a second eukaryotic selection agent, whereby said second eukaryotic selection agent is different to said first eukaryotic selection agent,

b1) providing a nucleic acid comprising
i) a prokaryotic origin of replication,
ii) a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,
iii) a second nucleic acid sequence encoding the heavy chain of said heterologous immunoglobulin, and/or a third nucleic acid sequence encoding the light chain of said heterologous immunoglobulin,

whereby a third transfection vector is provided which comprises said provided nucleic acid and an additional fourth nucleic acid sequence conferring resistance to a third eukaryotic selection agent, whereby said third eukaryotic selection agent is different to said first eukaryotic selection agent and is also different to said second eukaryotic selection agent,

c) transfecting said CHO cell, wherein said transfecting comprises the following:
(i) transfecting said CHO cell with said first transfection vector,
(ii) selecting a CHO cell transfected in (i) by selected growth in cultivation medium containing a first eukaryotic selection agent to which the first transfection vector confers resistance,
(iii) transfecting said selected CHO cell in (ii) with said second transfection vector,
(iv) selecting a CHO cell transfected in (iii) by selected growth in cultivation medium containing said first eukaryotic selection agent to which the first transfection vector confers resistance and said second eukaryotic selection agent to which the second transfection vector confers resistance,
(v) transfecting said CHO cell selected in (iv) with said third transfection vector,
(vi) selecting a CHO cell transfected in (v) by selected growth in a cultivation medium containing said first eukaryotic selection agent to which the first transfection vector confers resistance and said second eukaryotic selection agent to which the second transfection vector confers resistance and said third eukaryotic selection agent to which the third transfection vector confers resistance,

d) cultivating said transfected CHO cell in a medium in the presence of said first and said second and third eukaryotic selection agent, under conditions suitable for the expression of said second, and/or third nucleic acid, and

e) recovering said secreted heterologous immunoglobulin from the cultivation medium and thereby producing a heterologous immunoglobulin in a CHO cell which is secreted to the cultivation medium;

wherein said resultant CHO cell is stable in the absence of any or all selection agents, as used in the previous steps, for up to generation 60.

2. The method of claim **1**, wherein said CHO cell is selected from the group consisting of a CHO K1 cell, a CHO DG44 cell, a CHO XL99 cell, a CHO DXB11 cell, and a CHO DP12 cell; and wherein further the heterologous immunoglobulin is selected from the group consisting of an anti-AB

antibody, an anti P-selection antibody, an anti-IL-13R or antibody and an anti-CD4 antibody conjugate.

3. The method of claim **2**, wherein said second and/or third nucleic acid contains hybrid intronic nucleic acid sequence.

4. The method of claim **2**, characterized in that said first transfection vector and said second transfection vector differ only in the nucleic acid conferring resistance to said eukaryotic selection agent.

5. The method of claim **2**, wherein step c) and step d) are performed in the same medium.

6. The method of claim **5**, wherein said medium is selected form the group consisting of a serum-free medium, a serum-free medium supplemented with defined animal-derived components, an animal-derived component free medium, a protein-free medium, a protein-free medium supplemented with defined animal-derived components, a defined protein-free medium, and a chemically defined medium.

7. The method of, claim **2**, wherein the cultivating of step d) is either in the presence of the eukaryotic selection agents in a volume of less than 500 liter or said cultivating is in the absence of said eukaryotic selection agents in a volume of 500 liter or more, and that the recovering of the secreted heterologous immunoglobulin is from the cultivation medium without said eukaryotic selection agents.

8. The method of claim **2**, characterized in that the productivity of said CHO cells is over 40 generations not less than 70% and not more than 130% of the productivity after 10 generations of cultivation as split-batch cultivation.

9. The method of claim **8**, characterized in that the productivity of said CHO cell is at least 1.5 g/I of said heterologous immunoglobulin within 21 days as fed-batch cultivation.

10. The method of claim **1**, characterized in that said method further comprises:

f) purifying said heterologous immunoglobulin with one or more chromatographic steps.

11. The method of claim **10**, characterized in that said transfected CHO cell of step c) has

a doubling time of 150% or less of the doubling time of the CHO cell selected in substep (ii),

a volumetric yield of at least 125% compared to the volumetric yield of the CHO cell selected in (ii).

* * * * *

EXHIBIT EE

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US009080183B2

(12) **United States Patent**
Klein et al.

(10) **Patent No.: US 9,080,183 B2**
(45) **Date of Patent: Jul. 14, 2015**

(54) **PROMOTER**

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(73) Assignee: **HOFFMANN-LA ROCHE INC.**, Nutley, NJ (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 617 days.

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C12N 15/85 (2006.01)
C07K 14/47 (2006.01)

(52) **U.S. Cl.**

CPC ***C12N 15/85*** (2013.01); ***C07K 14/4702*** (2013.01)

(58) **Field of Classification Search**

None
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

2009/0041783 A1 * 2/2009 Takayama et al. 424/172.1

FOREIGN PATENT DOCUMENTS

JP 2006-050956 2/2006
WO WO 9962927 12/1999

OTHER PUBLICATIONS

Hartzell, S.W. et al., Proc. Natl. Acad. Sci 81:23-27 (1984).
(Translation of Korean Office Action 2009-70270412011).
(NCBI, GenBank, Accession No. EF550208.1 May 23,2007).
(Translation of Jap Off Act in Corres Jap Appl 2010513746 May 10, 2012).
Osamu Kanemitsu, Introduction to Antibody Engineering (Chijin Shokan Co., Ltd.),:198-201 (Jan. 25, 1994).
(Translation of Russian Off Act in Corres Appl 2010102812 Feb. 2, 2012).
Fromm M. et al: *Journal of Molecular and Applied Genetics*, vol. 1:5, (1982), pp. 457-481, XP008083910.
Fromm M. et al: *Journal of Molecular and Applied Genetics*, vol. 2:1, (1983), pp. 127-135, XP008083908.
Gorman C et al, *Molecular and Cellular Biology*, 2:9 (1982) 1044-105 XP000574017.
Dynan W et al, *Cll*, 35:1 (1983) 79-88 XP002451926.
Benoist C et al, *Nature*, 290:5804 (1981) 304-310 XP008083948.
Rio D et al, *Journal of Molecular and Applied Genetics*, 2:5 (1984) 423-435 XP008083924.
Everett R et al, *Nucleic Acids Research* 11:8 (1983) 2447-2464.
Firak T et al *Molecular and Cellular Biology*, 6:11, (1986) 3667-3676 XP002495690.
Gong, S et al, *Virology* 163:2 (1988) 481-493.

* cited by examiner

Primary Examiner — Celine Qian

(57) **ABSTRACT**

The current invention reports a promoter having the nucleic acid sequence of SEQ ID NO: 02, or SEQ ID NO: 03, or SEQ ID NO: 04, or SEQ ID NO: 06, which is a 5' shortened SV40 promoter with reduced promoter strength especially useful for the limited expression of heterologous polypeptides or selectable markers.

9 Claims, 7 Drawing Sheets

Fig. 1

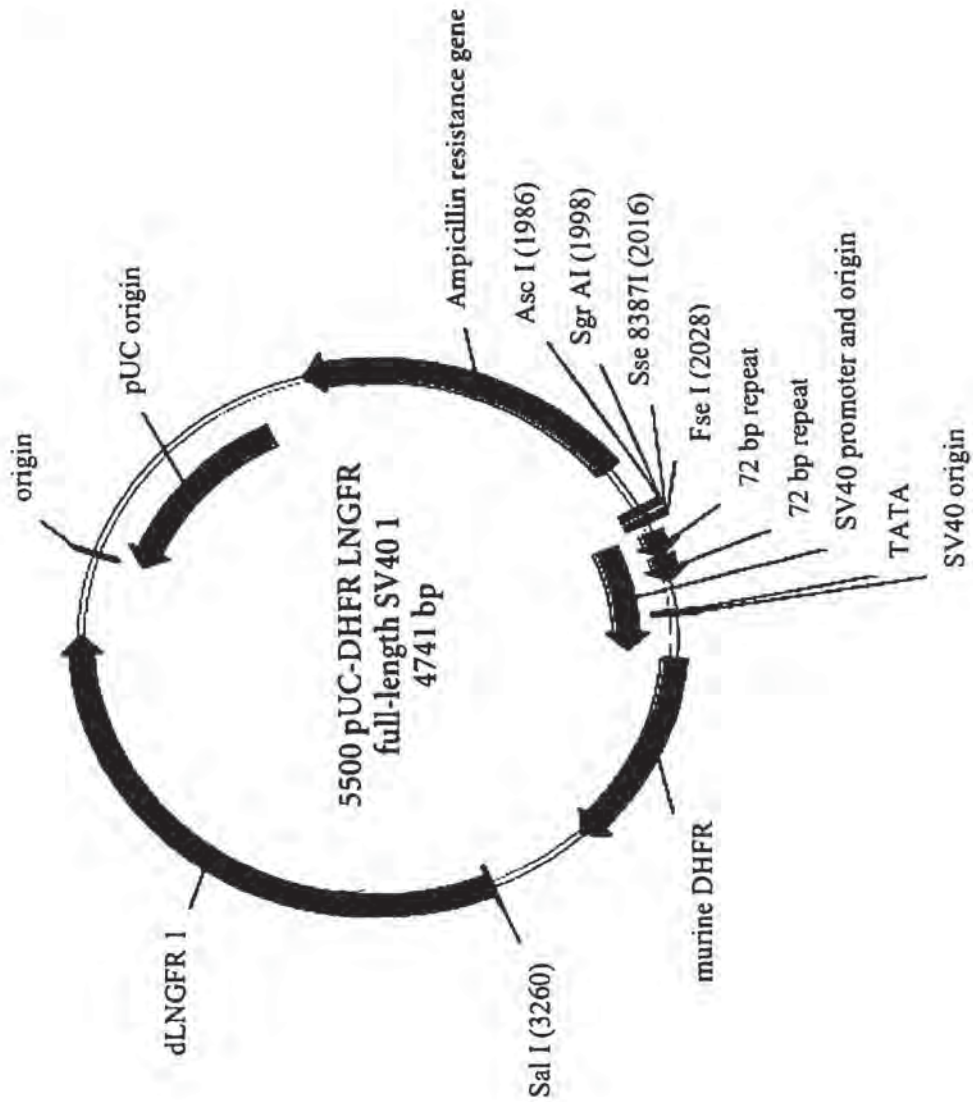


Fig. 2

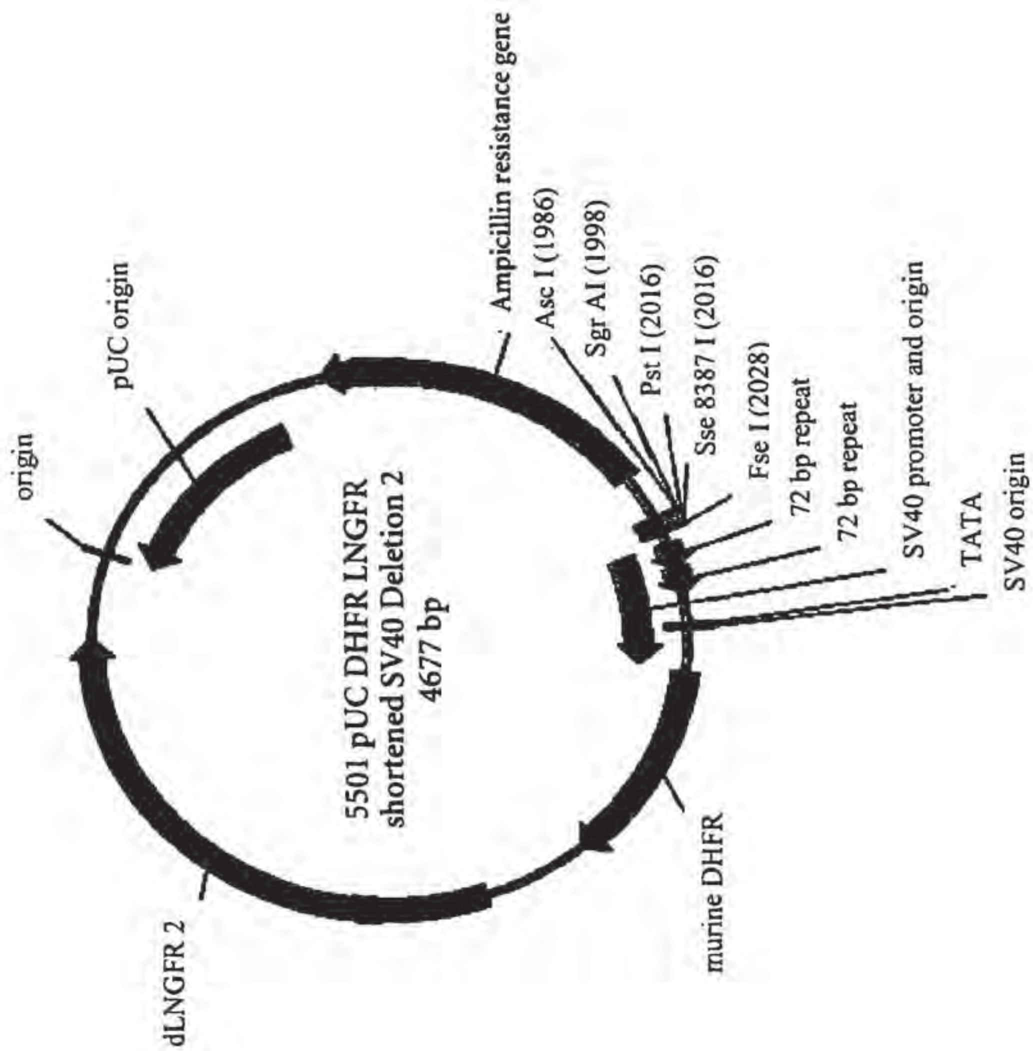


Fig. 3

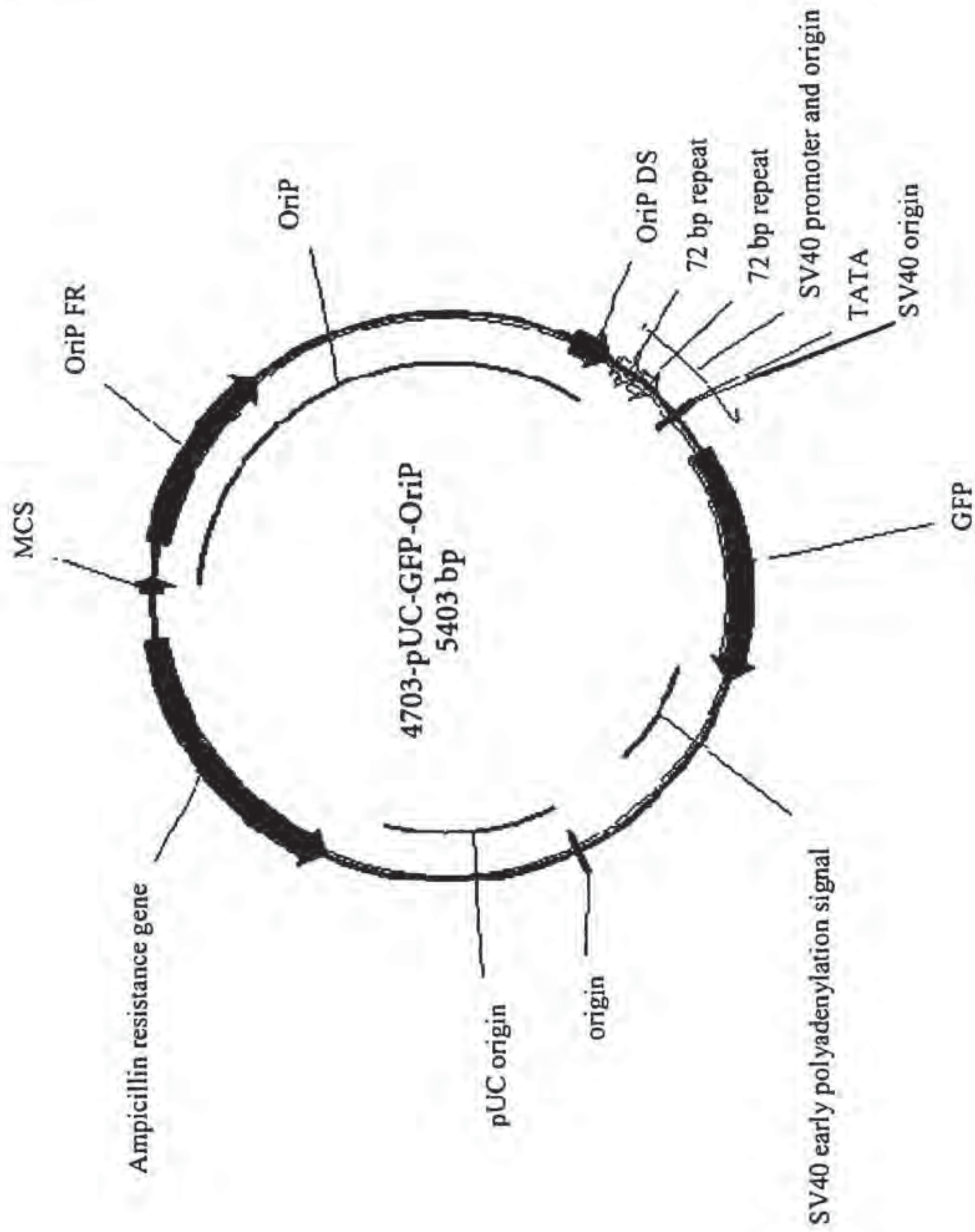


Fig. 4

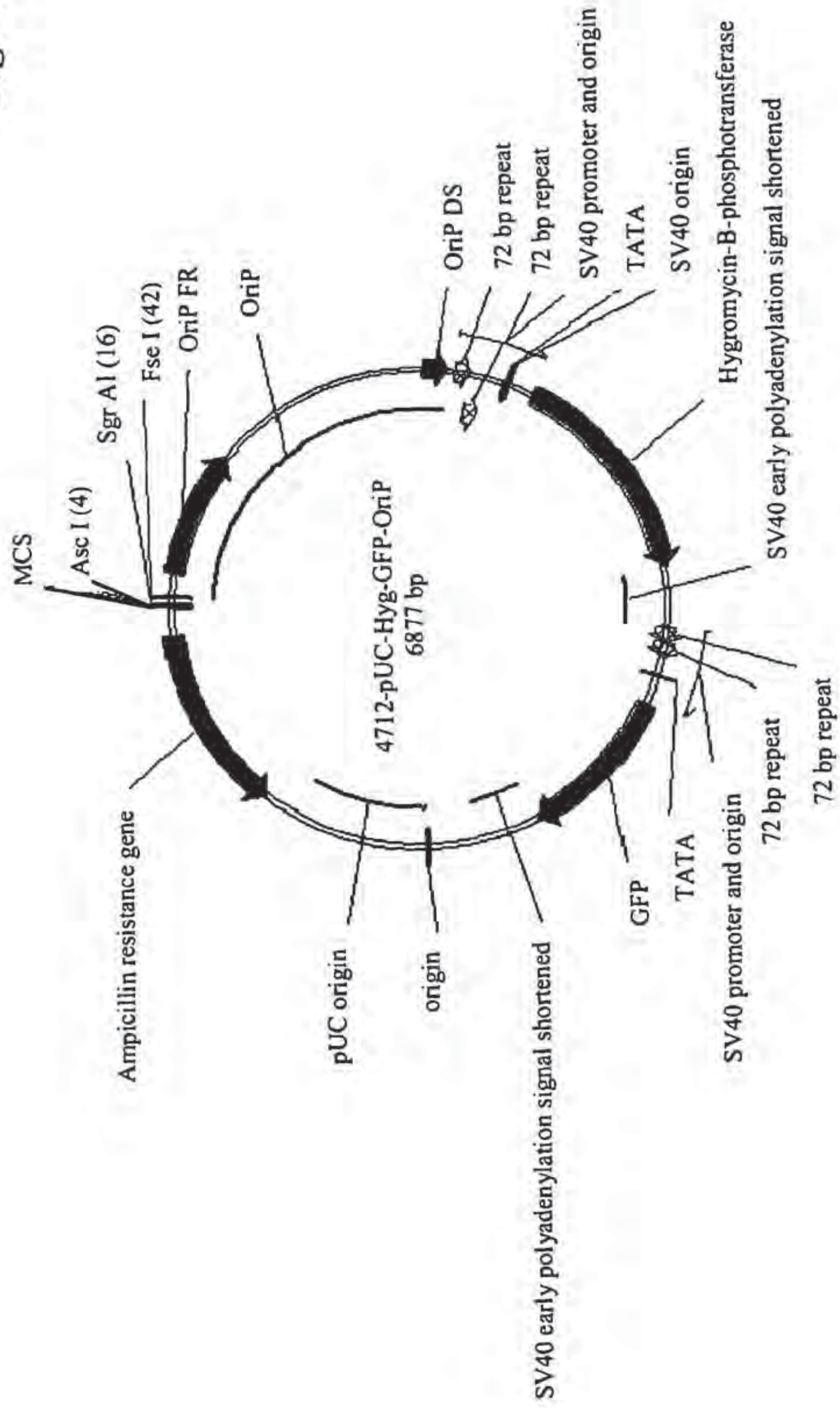


Fig. 5

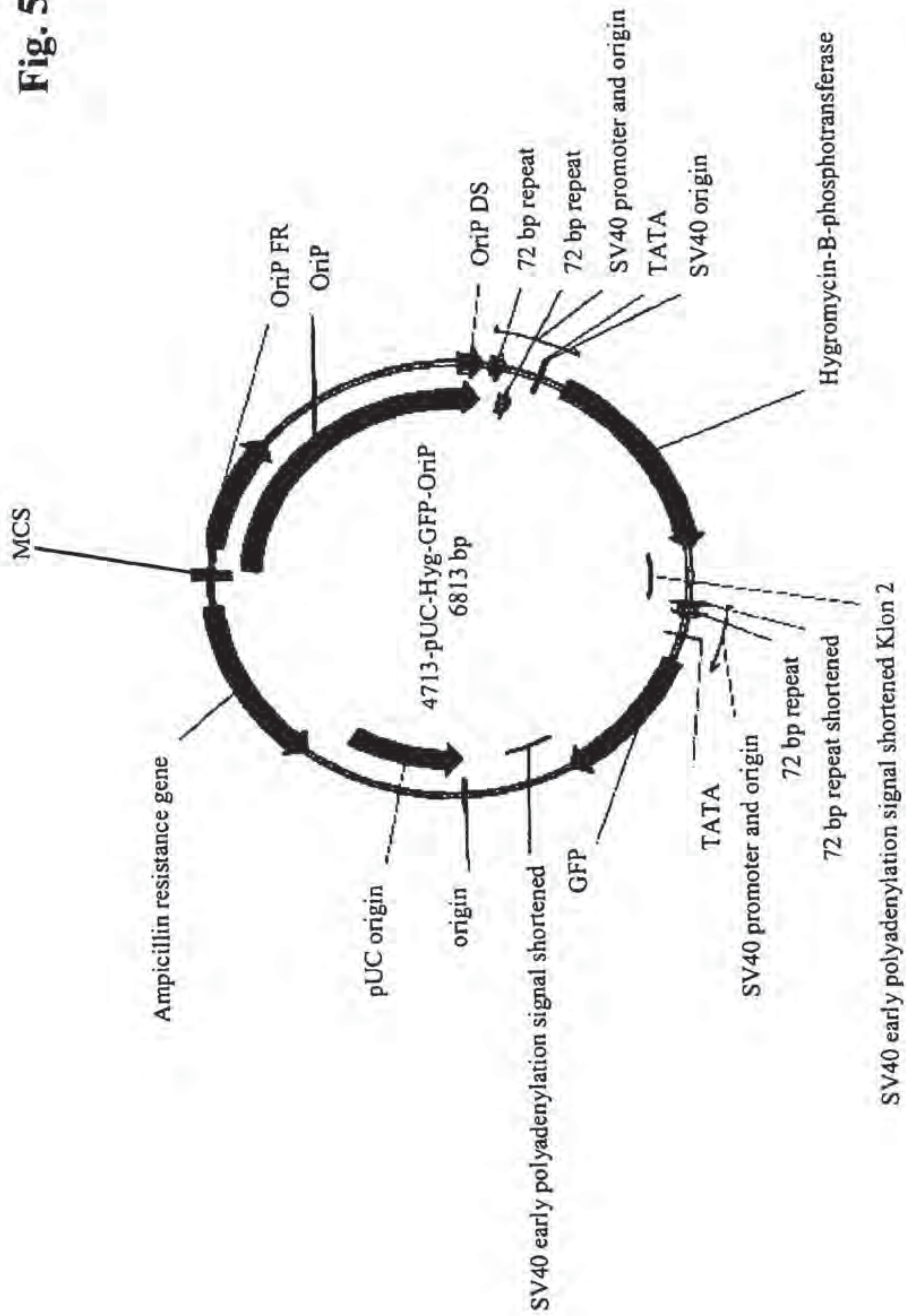


Fig. 6

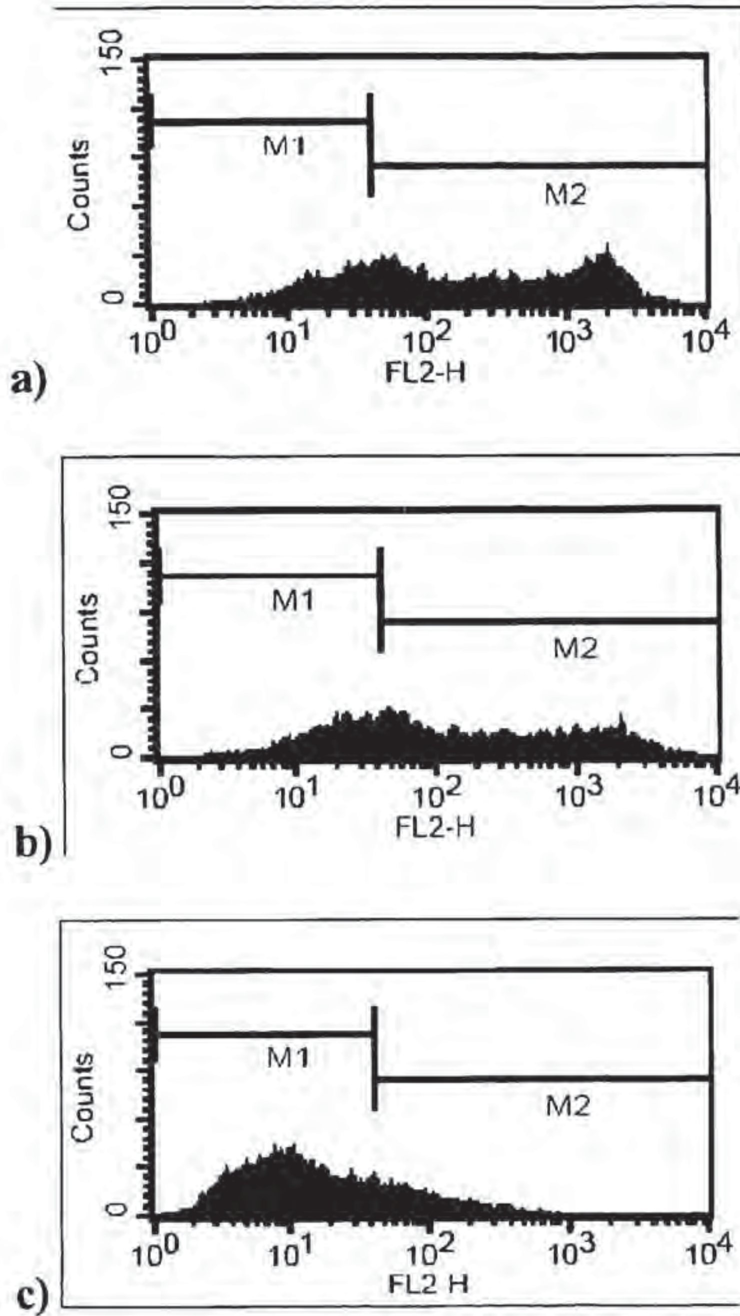
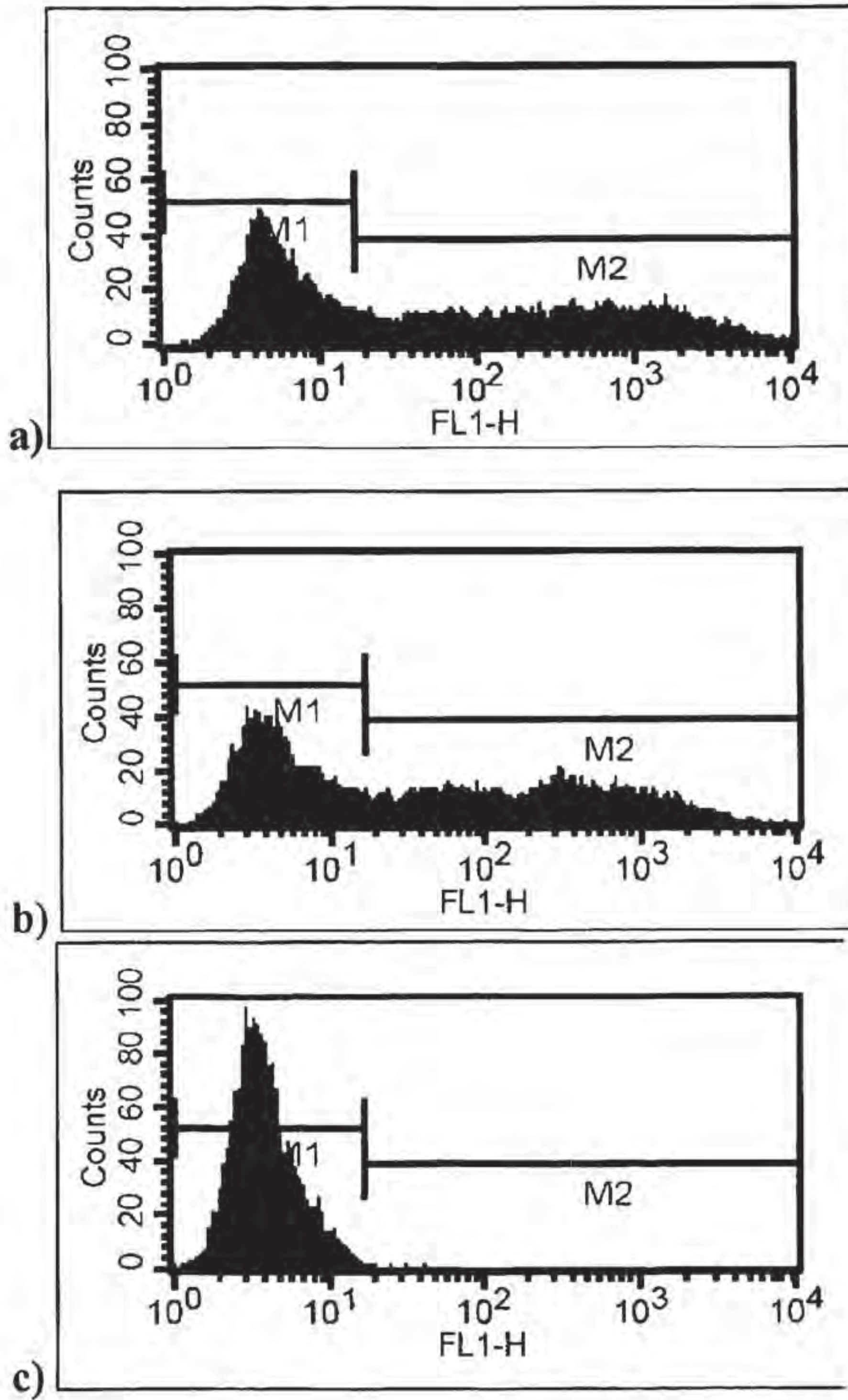


Fig. 7



PROMOTER

This application is the National Stage of International Application No. PCT/EP2008/005135 filed Jun. 25, 2008, which claims the benefit of EP 07012772.5 filed Jun. 29, 2007, which is hereby incorporated by reference in its entirety.

The current invention is in the field of protein expression and cell selection. It is herein reported a promoter with low promoter strength and thus with a limited expression of an operably linked coding nucleic acid.

BACKGROUND OF THE INVENTION

The expression of proteins is a fundamental process in living cells. All information required for protein expression is provided by a single nucleic acid. This nucleic acid not only contains the information of the protein's amino acid sequence, it also provides the regulatory information required (e.g. the ribosomal binding site, the start and end-signals for transcription, splice signals, enhancer elements, etc.) including a promoter/promoter sequence.

A promoter is a nucleic acid that regulates the amount of transcription of a nucleic acid, e.g. encoding a polypeptide, to which it is operably linked, into pre-mRNA. It is a transcription control element, which is located around the RNA polymerase initiation site at the 5'-end of an operably linked coding sequence. From analysis of the SV40 early promoter it is known that recognition/binding sites for transcription activators are contained in promoters in segments consisting of 7-20 basepairs. One segment is the start site for RNA synthesis, e.g. the well known TATA-box. Other segments, located approximately 30-110 basepairs 5', i.e. upstream, to the start site for RNA synthesis, are defining the frequency of transcription initiation. A promoter at least requires one segment that initiates RNA synthesis at a specific site and in a defined direction, i.e. in 5' to 3' direction.

Known promoters are the lac-lpp, the ara-, the lac-, the tac-, the trc-, the trp-, the phoA-, the P_{BAD}-, the λ_{PL}-, the lpp-, and the T7-promoter. The SV40 promoter is a nucleic acid sequence derived from the genome of Simian (vacuolating) Virus 40. For the recombinant production of a heterologous polypeptide in a eukaryotic or prokaryotic cell normally one or more expression plasmids are introduced into the cell. The expression plasmid(s) comprises an expression cassette for the expression of a heterologous polypeptide and also an expression cassette for the expression of a selectable marker, which is required for the selection of transfected cells expressing the heterologous polypeptide. The synthesis of the heterologous polypeptide and of the selectable marker both requires a fraction of the cell's expression machinery's capacity.

As it is the aim to produce predominantly the heterologous polypeptide most of the available capacity of the cell's expression machinery should be allocated to the expression of the nucleic acid encoding the heterologous polypeptide. Only a minor amount should be used for the expression of the selectable marker. This allocation of expression capacity is done via the strength of the corresponding promoters. The stronger a promoter is the more of the operably linked nucleic acid is transcribed and thus translated. Therefore, it exists a need for promoters with adjustable or reducible promoter strength.

Taylor, W. E., et al. (Endocrinol. 137 (1996) 5407-5414) report human stem cell factor promoter deletion variants. In US patent application US 2007/0092968 novel hTMC promoter and vectors for the tumor-selective and high-efficient

expression of cancer therapeutic genes is reported. Fromm et al. (J. Mol. Appl. Gen. 1 (1982) 457-481 and ibid 2 (1983) 127-135) report deletion mapping and deletion mutants of SV-40 early region promoter. Chitinase chitin-binding fragments are reported in U.S. Pat. No. 6,399,571. WO 99/62927 reports connective tissue growth factor-4.

SUMMARY OF THE INVENTION

The first aspect of the current invention is a promoter having, i.e. with, a nucleic acid sequence of SEQ ID NO: 02 or SEQ ID NO: 03 or SEQ ID NO: 04 or SEQ ID NO: 06. In one embodiment the promoter has the nucleic acid sequence of SEQ ID NO: 04.

A second aspect of the current invention is a nucleic acid that has the nucleotide sequence of SEQ ID NO: 04 and that has a promoter strength of 20% or less compared to the wild-type SV40 promoter of SEQ ID NO: 05 when operably linked to the nucleic acid sequence of SEQ ID NO: 07 encoding the green-fluorescent-protein (GFP).

A further aspect of the current invention is a method for the selection of a cell comprising the following steps in this order:

a) transfecting a eukaryotic cell with a nucleic acid comprising
   i) a first expression cassette comprising a nucleic acid encoding a heterologous polypeptide,
   ii) a second expression cassette comprising a first nucleic acid of SEQ ID NO: 04 and a second nucleic acid encoding a selectable marker, whereby the first nucleic acid is operably linked to the second nucleic acid,
b) cultivating said transfected cell under conditions suitable for growth of the non-transfected eukaryotic cell,
c) selecting a cell propagating in step b) and also
   i) propagating under selective culture conditions, or
   ii) expressing the selectable marker.

In one embodiment of this aspect of the invention the eukaryotic cell is a mammalian cell. In a preferred embodiment the mammalian cell is a CHO cell, BHK cell, or PER.C6® cell, or HEK cell, or Sp2/0 cell. In another embodiment the heterologous polypeptide is an immunoglobulin, or an immunoglobulin-fragment, or an immunoglobulin-conjugate. In one embodiment the selectable marker is a neomycin-aminoglycoside phosphotransferase, or a hygromycin-phosphotransferase, or dLNGFR, or GFP.

A forth aspect of the current invention is a method for the expression of a heterologous polypeptide which comprises the following steps in this order:

a) transfecting a mammalian cell with a nucleic acid comprising an expression cassette comprising a first nucleic acid of SEQ ID NO: 02 or SEQ ID NO: 03 or SEQ ID NO: 04 or SEQ ID NO: 06 operably linked to a second nucleic acid encoding a heterologous polypeptide,
b) selecting a cell transfected in step a),
c) cultivating the selected cell under conditions suitable for the expression of the heterologous polypeptide,
d) recovering the heterologous polypeptide from the cell or the cultivation medium.

In one embodiment of this aspect of the current invention the mammalian cell is a CHO cell, a BHK cell, or a PER.C6® cell, or HEK cell, or Sp2/0 cell. In another embodiment the first nucleic acid is of SEQ ID NO: 04. In a further embodiment the second nucleic acid is encoding an immunoglobulin, or an immunoglobulin-fragment, or an immunoglobulin-conjugate. In still another embodiment the nucleic acid comprises a second expression cassette encoding a selectable marker.

DETAILED DESCRIPTION OF THE INVENTION

The current invention reports a novel promoter nucleic acid with a nucleotide sequence of SEQ ID NO: 02, or SEQ ID NO: 03, or SEQ ID NO: 04, or SEQ ID NO: 06.

Methods and techniques useful for carrying out the current invention are known to a person skilled in the art and are described e.g. in Ausubel, F. M., ed., Current Protocols in Molecular Biology, Volumes I to III (1997), and Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). As known to a person skilled in the art enables the use of recombinant DNA technology the production of numerous derivatives of a nucleic acid and/or polypeptide. Such derivatives can, for example, be modified in one individual or several positions by substitution, alteration, exchange, deletion, or insertion. The modification or derivatisation can, for example, be carried out by means of site directed mutagenesis. Such modifications can easily be carried out by a person skilled in the art (see e.g. Sambrook, J., et al., Molecular Cloning: A laboratory manual (1999) Cold Spring Harbor Laboratory Press, New York, USA). The use of recombinant technology enables a person skilled in the art to transform various host cells with heterologous nucleic acid(s).

A "promoter" refers to a nucleic acid, i.e. polynucleotide sequence, which controls transcription of a nucleic acid to which it is operably linked. A promoter may include signals for RNA polymerase binding and transcription initiation. The promoter(s) used will be functionable in the cell type of the host cell in which expression of the operably linked nucleic acid is contemplated. A large number of promoters including constitutive, inducible, and repressible promoters from a variety of different sources are well known in the art (and identified in databases such as GenBank). They are available as or within cloned polynucleotides (from, e.g., depositories such as ATCC as well as other commercial or individual sources). A "promoter" comprises a nucleotide sequence that directs the transcription of e.g. an operably linked structural gene. Typically, a promoter is located in the 5' non-coding or 5'-untranslated region (5'UTR) of a gene, proximal to the transcriptional start site of a structural gene. Sequence elements within promoters that function in the initiation of transcription are often characterized by consensus nucleotide sequences. These sequence elements include RNA polymerase binding sites, TATA sequences, CAAT sequences, differentiation-specific elements (DSEs; McGehee, R. E., et al., Mol. Endocrinol. 7 (1993) 551), cyclic AMP response elements (CREs), serum response elements (SREs; Treisman, R., Seminars in Cancer Biol. 1 (1990) 47), glucocorticoid response elements (GREs), and binding sites for other transcription factors, such as CRE/ATF (O'Reilly, M. A., et al., J. Biol. Chem. 267 (1992) 19938), AP2 (Ye, J., et al., J. Biol. Chem. 269 (1994) 25728), SP1, cAMP response element binding protein (CREB; Loeken, M. R., Gene Expr. 3 (1993) 253-264) and octamer factors (see, in general, Watson et al., eds., Molecular Biology of the Gene, 4th ed., The Benjamin/Cummings Publishing Company, Inc. 1987, and Lemaigre, F. P. and Rousseau, G. G., Biochem. J. 303 (1994) 1-14). If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter. Repressible promoters are also known. For example, the c-fos promoter is specifically activated upon binding of growth hormone to its receptor on the cell surface. Tetracycline (tet) regulated expression can be achieved by artificial hybrid promoters that consist e.g. of a CMV promoter followed by two Tet-operator sites. The Tet-repressor binds to the two Tet-operator sites and blocks transcription. Upon addition of the inducer tetracycline, the Tet-repressor is released from the Tet-operator sites and transcription proceeds (Gossen, M. and Bujard, H., Proc. Natl. Acad. Sci. USA 89 (1992) 5547-5551). For other inducible promoters including metallothionein and heat shock promoters, see, e.g., Sambrook, et al. (supra), and Gossen, M., et al., Curr. Opin. Biotech. 5 (1994) 516-520. Among the eukaryotic promoters that have been identified as strong promoters for high-level expression are the SV40 early promoter, adenovirus major late promoter, mouse metallothionein-I promoter, Rous sarcoma virus long terminal repeat, Chinese hamster elongation factor 1 alpha (CHEF-1, see e.g. U.S. Pat. No. 5,888,809), human EF-1 alpha, ubiquitin, and human cytomegalovirus immediate early promoter (CMV IE). An enhancer (i.e., a cis-acting DNA element that acts on a promoter to increase transcription) may be necessary to function in conjunction with the promoter to increase the level of expression obtained with a promoter alone, and may be included as a transcriptional regulatory element. Often, the polynucleotide segment containing the promoter will include enhancer sequences as well (e.g., CMV or SV40).

The term "nucleic acid" as used herein, is a polymer consisting of individual nucleotides, i.e. a polynucleotide. It refers to a naturally occurring, or partially or fully non-naturally occurring nucleic acid, which is e.g. encoding a polypeptide that can be produced recombinantly. The nucleic acid can be build up of DNA-fragments which are either isolated or synthesized by chemical means. The nucleic acid can be integrated into another nucleic acid, e.g. in an expression plasmid or the genome/chromosome of a host cell. Plasmid includes shuttle and expression vectors. Typically, the plasmid will also comprise a prokaryotic propagation unit comprising an origin of replication (e.g. the ColE1 origin of replication) and a selectable marker (e.g. ampicillin or tetracycline resistance gene) for replication and selection, respectively, of the vector in bacteria.

The term "promoter strength" and grammatical equivalents thereof as used within the current invention denotes the efficacy of a promoter in the transcription of an operably linked nucleic acid. The promoter strength of a promoter can be high, i.e. it can be of from 90% to more than 100%, or medium, i.e. it can be of from 40% to less than 90%, or low, i.e. it can be up to less than 40%, if compared to the promoter strength of the wild-type SV40 promoter of SEQ ID NO: 05. This value can be determined by comparing the amount of expression of a heterologous polypeptide operably linked to the promoter in question to the amount of expression of the heterologous polypeptide operably linked to the wild-type SV40 promoter in the same cell type. This can be done e.g. by determining the amount of expression of the heterologous polypeptide in a CHO- or HEK-cell transfected with an expression cassette consisting of the promoter in question operably linked to a nucleic acid encoding the heterologous polypeptide by an ELISA-assay. By comparing this amount to the amount of expression of the same heterologous polypeptide in the same cell line transfected with an expression cassette consisting of the wild-type SV40 promoter operably linked to a nucleic acid encoding the heterologous polypeptide determined with the same ELISA-assay i.e. comparing the amount of heterologous polypeptide in the same cell with the same expression plasmid wherein only the promoter is changed, the relative promoter strength can be determined. The term "wild-type SV40 promoter" as used within this application denotes a nucleic acid of SEQ ID NO: 05

which correspond to position 72-411 of the nucleic acid of SEQ ID NO: 01, which is the genome of the SV40.

"Operably linked" refers to a juxtaposition of two or more components, wherein the components so described are in a relationship permitting them to function in their intended manner. For example, a promoter and/or enhancer are operably linked to a coding sequence, if it acts in cis to control or modulate the transcription of the linked coding sequence. Generally, but not necessarily, the DNA sequences that are "operably linked" are contiguous and, where necessary to join two protein encoding regions such as a secretory leader/signal sequence and a polypeptide, contiguous and in reading frame. However, although an operably linked promoter is generally located upstream of the coding sequence, it is not necessarily contiguous with it. Enhancers do not have to be contiguous. An enhancer is operably linked to a coding sequence if the enhancer increases transcription of the coding sequence. Operably linked enhancers can be located upstream, within, or downstream of coding sequences, and at considerable distance from the promoter. A polyadenylation site is operably linked to a coding sequence if it is located at the downstream end of the coding sequence in such a way that transcription proceeds through the coding sequence into the polyadenylation sequence. Linking is accomplished by recombinant methods known in the art, e.g., using PCR methodology, and/or by ligation at convenient restriction sites. If convenient restriction sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

Within the scope of the present invention, transfected cells may be obtained with substantially any kind of transfection method known in the art. For example, the nucleic acid may be introduced into the cells by means of electroporation or microinjection. Alternatively, lipofection reagents such as FuGENE 6 (Roche Diagnostics GmbH, Germany), X-tremeGENE (Roche Diagnostics GmbH, Germany), and LipofectAmine (Invitrogen Corp., USA) may be used. Still alternatively, the nucleic acid may be introduced into the cell by appropriate viral vector systems based on retroviruses, lentiviruses, adenoviruses, or adeno-associated viruses (Singer, O., Proc. Natl. Acad. Sci. USA 101 (2004) 5313-5314).

The term "cell" or "host cell" refers to a cell into which a nucleic acid, e.g. encoding a heterologous polypeptide or constituting an shRNA, can be or is introduced/transfected. Host cells include both prokaryotic cells, which are used for propagation of vectors/plasmids, and eukaryotic cells, which are used for the expression of the nucleic acid. In one embodiment the eukaryotic cells are mammalian cells. In another embodiment the mammalian host cell is selected from the mammalian cells comprising CHO cells (e.g. CHO K1 or CHO DG44), BHK cells, NS0 cells, SP2/0 cells, HEK 293 cells, HEK 293 EBNA cells, PER.C6 cells, and COS cells. In a further embodiment the mammalian cell is selected from the group comprising hybridoma, myeloma, and rodent cells. Myeloma cells comprise rat myeloma cells (e.g. YB2), and mouse myeloma cells (e.g. NS0, SP2/0). Polypeptides for use in pharmaceutical applications are in one embodiment produced in mammalian cells such as CHO cells, NS0 cells, Sp2/0 cells, COS cells, HEK cells, BHK cells, PER.C6® cells, or the like. For the fermentation of the host cell and thus for the expression of the polypeptide of interest a cultivation medium is used. Today CHO cells are widely used for the expression of pharmaceutical polypeptides, either at small scale in the laboratory or at large scale in production processes. Due to their wide distribution and use the characteristic properties and the genetic background of CHO cells is well known. Therefore, CHO cells are approved by regulatory

authorities for the production of therapeutic proteins for application to human beings. In one embodiment the mammalian cell is a CHO cell.

An "expression cassette" refers to a nucleic acid that contains the elements necessary for expression and secretion of at least the contained structural gene in a host cell. A nucleic acid is likewise characterized by its sequence consisting of individual nucleotides or by the amino acid sequence encoded by the nucleic acid molecule.

A "gene" denotes a nucleic acid which is a segment e.g. on a chromosome or on a plasmid which can effect the expression of a peptide, polypeptide, or protein. Beside the coding region, i.e. the structural gene, a gene comprises other functional elements e.g. a signal sequence, promoter(s), introns, and/or terminators.

A "structural gene" denotes the region of a gene without a signal sequence, i.e. the coding region.

The term "expression" as used herein refers to transcription and/or translation occurring within a cell. The level of transcription of a desired product in a host cell can be determined on the basis of the amount of corresponding mRNA that is present in the cell. For example, mRNA transcribed from a selected nucleic acid can be quantitated by PCR or by Northern hybridization (see Sambrook, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989)). The protein encoded by a selected nucleic acid can be quantitated by various methods, e.g. by ELISA, by assaying for the biological activity of the protein, or by employing assays that are independent of such activity, such as Western blotting or radioimmunoassay, by using antibodies that recognize and bind to the protein (see Sambrook, et al., 1989, supra).

"Regulatory elements" as used herein, refer to nucleotide sequences present in cis, necessary for transcription and/or translation of the nucleic acid sequence encoding a polypeptide of interest. The transcriptional regulatory elements normally comprise a promoter upstream of the nucleic acid sequence to be expressed, transcriptional initiation and termination sites, and a polyadenylation signal sequence. The term "transcriptional initiation site" refers to the nucleotide in the nucleic acid corresponding to the first nucleotide incorporated into the primary transcript, i.e. the mRNA precursor; the transcriptional initiation site may overlap with the promoter sequence. The term "transcriptional termination site" refers to a nucleotide sequence normally represented at the 3' end of a gene of interest to be transcribed, that causes RNA polymerase to terminate transcription. The polyadenylation signal sequence, or poly-A addition signal provides the signal for the cleavage at a specific site at the 3' end of eukaryotic mRNA and the post-transcriptional addition in the nucleus of a sequence of about 100-200 adenine nucleotides (polyA tail) to the cleaved 3' end. The polyadenylation signal sequence may include the consensus sequence AATAAA located at about 10-30 nucleotides upstream from the site of cleavage.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 20 amino acid residues may be referred to as "peptides." Polypeptides comprising two or more amino acid chains or comprising an amino acid chain of a length of 100 amino acids or more may be referred to as "proteins". A polypeptide or protein may also comprise non-peptidic components, such as carbohydrate groups or metal ions. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and may vary with the type of cell. Proteins and polypeptides are defined herein in terms of their amino acid

backbone structure; additions such as carbohydrate groups are generally not specified, but may be present nonetheless.

"Heterologous DNA" or "heterologous polypeptide" refers to a DNA molecule or a polypeptide, or a population of DNA molecules or a population of polypeptides, that do not exist naturally within a given host cell. DNA molecules heterologous to a particular host cell may contain DNA derived from the host cell species (i.e. endogenous DNA) so long as that host cell derived DNA is combined with non-host cell derived DNA (i.e. exogenous DNA). For example, a DNA molecule containing a non-host DNA segment encoding a polypeptide operably linked to a host DNA segment comprising a promoter is considered to be a heterologous DNA molecule. Conversely, a heterologous DNA molecule can comprise an endogenous structural gene operably linked with an exogenous promoter. A peptide or polypeptide encoded by a non-host DNA molecule is a "heterologous" peptide or polypeptide.

The term "selectable marker" denotes a nucleic acid that allows cells carrying this nucleic acid to be specifically selected for or against, in the presence of a corresponding "selection agent". A useful positive selectable marker is e.g. an antibiotic resistance gene. The selectable marker allows a cell which is transformed therewith to be selected for in the presence of the corresponding selection agent; a non-transformed cell is not capable to grow or survive under selective culture conditions, i.e. in the presence of the selection agent. Selectable markers can be positive, negative or bifunctional. Positive selectable markers allow the selection of cells carrying the marker, whereas negative selectable markers allow cells carrying the marker to be selectively eliminated. Typically, a selectable marker will confer resistance to a drug or compensate for a metabolic or catabolic defect in the cell. Selectable markers useful with eukaryotic cells include, e.g., the genes for aminoglycoside phosphotransferase (APH), such as the hygromycin phosphotransferase (HYG), neomycin and G418 APH, dihydrofolate reductase (DHFR), thymidine kinase (TK), glutamine synthetase (GS), asparagine synthetase, tryptophan synthetase (selection agent indole), histidinol dehydrogenase (selection agent histidinol D), and genes providing resistance to puromycin, bleomycin, phleomycin, chloramphenicol, Zeocin, and mycophenolic acid. Further selectable markers are reported in WO 92/08796 and WO 94/28143.

The term "expression machinery" as used within the current invention denotes the sum of the enzymes, cofactors, etc. of a cell, which are involved in the process beginning with the transcription of a nucleic acid or gene (i.e. also called "gene expression machinery") to the post-translational modification of the polypeptide encoded by the nucleic acid. The "expression machinery" e.g. comprises the steps of transcription of DNA into pre-mRNA, pre-mRNA splicing to mature mRNA, translation of the mRNA into a polypeptide, and post translational modification of the polypeptide.

The term "under conditions suitable for the expression of a heterologous polypeptide" denotes conditions which are used for the cultivation of a mammalian cell expressing a heterologous polypeptide and which are known to or can easily be determined by a person skilled in the art. It is also known to a person skilled in the art that these conditions may vary depending on the type of mammalian cell cultivated and type of protein expressed. In general the mammalian cell is cultivated at a temperature, e.g. between 20° C. and 40° C., and for a period of time sufficient to allow effective protein production, e.g. for 4 to 28 days, in a volume of from 0.1 liter to 10^7 liter.

The term "under conditions suitable for the growth of the non-transfected cell" denotes conditions which are generally used for the cultivation of a non-transfected cell of the same cell line. These conditions are known or can easily be determined by a person skilled in the art.

The term "recovering of the heterologous polypeptide" as used within the current application denotes precipitation, salting out, ultrafiltration, diafiltration, lyophilization, solvent volume reduction to obtain a concentrated solution, or chromatography. Generally chromatographic processes are used for the separation and purification of polypeptides. Different methods are well established and widespread used for protein recovery and purification, such as affinity chromatography with microbial proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and electrophoretical methods (such as gel electrophoresis, capillary electrophoresis) (Vijayalakshmi, M. A., Appl. Biochem. Biotech. 75 (1998) 93-102).

The term "immunoglobulin" refers to a protein consisting of one or more polypeptide(s) substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the different constant region genes as well as the myriad immunoglobulin variable region genes. Immunoglobulins may exist in a variety of formats, including, for example, Fv, Fab, and F(ab)2 as well as single chains (scFv) or diabodies (e.g. Huston, J. S., et al., Proc. Natl. Acad. Sci. USA 85 (1988) 5879-5883; Bird, R. E., et al., Science 242 (1988) 423-426; in general, Hood, et al., Immunology, Benjamin N.Y., 2nd edition (1984); and Hunkapiller, T. and Hood, L., Nature 323 (1986) 15-16).

An immunoglobulin in general comprises two so called light chain polypeptides (light chain) and two so called heavy chain polypeptides (heavy chain). Each of the heavy and light chain polypeptides contains a variable domain (variable region) (generally the amino terminal portion of the polypeptide chain) comprising binding regions that are able to interact with an antigen. Each of the heavy and light chain polypeptides comprises a constant region (generally the carboxyl terminal portion). The constant region of the heavy chain mediates the binding of the antibody i) to cells bearing a Fc gamma receptor (FcγR), such as phagocytic cells, or ii) to cells bearing the neonatal Fc receptor (FcRn) also known as Brambell receptor. It also mediates the binding to some factors including factors of the classical complement system such as component (Clq). The variable domain of an immunoglobulin's light or heavy chain in turn comprises different segments, i.e. four framework regions (FR) and three hypervariable regions (CDR).

An "immunoglobulin fragment" denotes a polypeptide comprising at least one domain of the group of domains comprising the variable domain, the C_H1 domain, the hinge-region, the C_H2 domain, the C_H3 domain, the C_H4 domain of a heavy chain of an immunoglobulin or the variable domain or the C_L domain of a light chain of an immunoglobulin. Also comprised are derivatives and variants thereof. Additionally a variable domain, in which one or more amino acids or amino acid regions are deleted, may be present.

An "immunoglobulin conjugate" denotes a polypeptide comprising at least one domain of an immunoglobulin heavy

or light chain conjugated via a peptide bond to a further polypeptide. The further polypeptide is a non-immunoglobulin peptide, such as a hormone, growth receptor, antifusogenic peptide or the like.

The current invention reports a promoter with a nucleotide sequence of SEQ ID NO: 02, or SEQ ID NO: 03, or SEQ ID NO: 04, or SEQ ID NO: 06

A method for the identification of a potential high producer cell clone is the linking of the expression of a selectable marker gene and a structural gene encoding a heterologous polypeptide via an internal ribosome entry site (IRES). With this design the expression of the heterologous polypeptide can be correlated with the expression of the selectable marker. Another method is gene amplification. Therein cells deficient of the enzyme dihydrofolate reductase (DHFR) are transfected with a vector/plasmid which contains a first expression cassette for the expression of the DHFR protein and a second expression cassette for the expression of a heterologous polypeptide. By using a culture medium depleted of glycine, hypoxanthine and thymidine selective culture conditions are established. For amplification a DHFR inhibitor, methotrexate (MTX), is added (Kaufman, R. J., et al., J Mol. Biol. 159 (1982) 601-621; U.S. Pat. No. 4,656,134). Generally may be used any kind of gene whose expression product is located/can be detected on the cell surface as a marker for enrichment and selection of transfectants. dLNGFR, a truncated form of the low-affinity nerve growth factor receptor, and thus inactive for signal transduction, which is expressed on the cell surface, and has proven to be a highly useful marker for cell biological analysis (Philipps, K., et al., Nat. Med. 2 (1996) 1154-1156 and Machl, A. W., et al., Cytometry 29 (1997) 371-374).

In order not to unnecessarily reduce the production of a heterologous polypeptide of interest the expression of the selectable marker, which is required for the selection of cells producing the heterologous polypeptide, i.e. of successfully transfected cells, should be as low as possible but nonetheless still detectable.

It has now surprisingly been found that this need can be fulfilled with a promoter according to the invention. By employing a promoter according to the current invention cells can be selected which express a heterologous polypeptide at a higher level compared to cells selected under the same conditions and not employing a promoter according to the current invention. It has surprisingly been found that with a promoter according to the invention a cell expressing a heterologous polypeptide can be isolated with reduced expenditure. Additionally it has been found that by employing a promoter according to the current invention cells can be selected that express a heterologous polypeptide at a higher level compared to cells selected by employing a full length SV40 promoter under the same conditions and selection agent concentrations.

The term "5' shortened SV40 promoter" as used within the current application denotes a wild-type SV40 promoter in which a defined number of consecutive nucleotides at the 5' end of the nucleic acid sequence have been deleted.

Thus, the current invention reports a promoter having, i.e. with, the nucleic acid sequence of SEQ ID NO: 02. SEQ ID NO: 02 comprises nucleotides 61 to 348 of the wild-type SV40 promoter of SEQ ID NO: 05, i.e. nucleotides 1 to 60 have been deleted. The preparation of the promoter with SEQ ID NO: 02 is shown in Example 1.

The current invention also reports a promoter having, i.e. with, the nucleic acid sequence of SEQ ID NO: 03. SEQ ID NO: 03 corresponds to nucleotides 130 to 348 of the wild-type SV40 promoter of SEQ ID NO: 05, i.e. nucleotides 1 to 129 have been deleted. The preparation of the promoter with SEQ ID NO: 03 is shown in Example 2.

The current invention finally reports a promoter having, i.e. with, the nucleic acid sequence of SEQ ID NO: 04. SEQ ID NO: 04 is nucleotides 177 to 348 of the wild-type SV40 promoter of SEQ ID NO: 05, i.e. nucleotides 1 to 176 have been deleted. The preparation of the promoter with SEQ ID NO: 04 is shown in Example 3.

The current invention finally reports a promoter having, i.e. with, the nucleic acid sequence of SEQ ID NO: 06. SEQ ID NO: 06 consists of nucleotides 203 to 348 of the wild-type SV40 promoter of SEQ ID NO: 05, i.e. nucleotides 1 to 202 have been deleted.

To determine the promoter strength of the promoters with a nucleic acid sequence of SEQ ID NO: 02 to 04 and 06 expression plasmids have been generated in which each of the different promoters is operably linked to a nucleic acid encoding GFP (green fluorescent protein, SEQ ID NO: 07). As can be seen from FIGS. 7 *a*) to *c*) the 5' deletion of nucleotides in the wild-type SV40 promoter nucleic acid reduces the promoter strength. The promoter of SEQ ID NO: 02 has approximately the same strength as the full-length wild-type SV40 promoter. The promoters of SEQ ID NO: 03 and 04 have promoter strength of approximately 56% and approximately 19%, respectively. Thus with the promoters according to the current invention the expression of a nucleic acid operably linked thereto can be reduced or limited compared to the wild-type SV40 promoter.

In simian virus 40 is the SV40 promoter preceded by two 72 bp repeats. In one embodiment of the current invention is the first 72 bp repeat deleted and the second 72 bp repeat maintained. In one embodiment the nucleic acid according to the invention comprises the nucleic acid of SEQ ID NO: 14 prior to the nucleic acid of SEQ ID NO: 04. In another embodiment the nucleic acid according to the invention comprises the second 72 bp repeat of SEQ ID NO: 14 of the simian virus 40 promoter. In a further embodiment in the nucleic acid according to the invention the first 72 bp repeat of the SV40 promoter is deleted and the second 72 bp repeat of the SV40 promoter is maintained. This is useful for the expression of a heterologous polypeptide. In this embodiment is the promoter according to the current invention, which is only containing the second 72 bp repeat of the wild type SV40 promoter, operably linked to a nucleic acid encoding a selectable marker. With the reduced promoter strength of this promoter the expression of the selectable marker is reduced whereas the expression of the heterologous polypeptide is maintained by using e.g. the wild type SV40 promoter of SEQ ID NO: 05. Thus, it has been found that the combination of a promoter according to the invention operably linked to a nucleic acid encoding a selectable marker and of a wild-type promoter, e.g. SV40 or CMV, operably linked to a nucleic acid encoding a heterologous polypeptide of interest results in an improved expression of the heterologous polypeptide compared to constructs in which the nucleic acid encoding a selectable marker as well as the nucleic acid encoding the heterologous polypeptide of interest are both operably linked to a wild-type promoter.

In stable cell clones the nucleic acid encoding the selectable marker and the nucleic acid encoding the heterologous polypeptide as well as their corresponding promoters are integrated jointly in the genome of said cell. As the location of the integration in the genome is a random process a selection step is normally carried out. In this selection step only cells are selected in which the joint nucleic acids are incorporated in the genome is close proximity of a transcriptionally highly active locus. Cells either having incorporated the nucleic acid

afar from such a locus or having incorporated both nucleic acids at different loci are eliminated by the selection step.

Another aspect of the current invention is a nucleic acid consisting of a nucleic acid sequence of SEQ ID NO: 02, or SEQ ID NO: 03, or SEQ ID NO: 04, or SEQ ID NO: 06, which has a promoter strength of 90% or more, or 40% to less than 90%, or less than 40% of the promoter strength of the wild-type SV40 promoter of SEQ ID NO: 05 when operably linked to the nucleic acid of SEQ ID NO: 07.

In a preferred embodiment has the nucleic acid the nucleic acid sequence of SEQ ID NO: 04 and a promoter strength of 20% or less of the promoter strength of the wild-type SV40 promoter of SEQ ID NO: 05 when each of them individually is operably linked to the nucleic acid of SEQ ID NO: 07.

As the nucleic acid and the promoter, respectively, according to the invention each has reduced promoter strength, i.e. a nucleic acid operably linked thereto is transcribed at a reduced amount or with a reduced rate when compared to the wild-type SV40 promoter, they are useful in multiple applications.

For example, they can be used to promote the expression of an operably linked selection marker allowing for the selection of a cell carrying this selection marker without requiring a large fraction of the capacity of the cell's protein expression machinery. Thereby the expression of an e.g. co-expressed heterologous polypeptide is not negatively affected.

Another aspect of the current invention is a method for the selection of a cell expressing a heterologous polypeptide comprising the steps of

a) transfecting a eukaryotic cell with a nucleic acid comprising
   i) a first expression cassette comprising a nucleic acid encoding a heterologous polypeptide,
   ii) a second expression cassette comprising a first nucleic acid of SEQ ID NO: 04 and a second nucleic acid encoding a selectable marker, whereby the first nucleic acid is operably linked to the second nucleic acid,
b) cultivating said transfected cell under conditions suitable for growth of the non-transfected eukaryotic cell,
c) selecting a cell propagating in step b) and also
   i) propagating under selection condition, or
   ii) expressing the selectable marker.

Cells suitable in this method are e.g. CHO cells, BHK cells, PER.C6® cells, HEK cells, HeLa cells, SP2/0 cells, NS0 cells, myeloma cells, or hybridoma cells. In one embodiment the cell is a mammalian cell, in a preferred embodiment the cell is selected from a CHO cell, BHK cell, HEK cell, Sp2/0 cell, or a PER.C6® cell.

The heterologous polypeptide may be any heterologous polypeptide of interest, such as e.g. prodrugs, enzymes, enzyme fragments, enzyme inhibitors, enzyme activators, biologically active polypeptides, hedgehog proteins, bone morphogenetic proteins, growth factors, erythropoietin, thrombopoietin, G-CSF, interleukins, interferons, immunoglobulins, or antifusogenic peptides, or fragments thereof, or conjugates thereof. In one embodiment the heterologous polypeptide is an immunoglobulin, or an immunoglobulin fragment, or an immunoglobulin conjugate.

In one embodiment step c) of the method is selecting a cell propagating in step b) under selective culture conditions, i.e. in the presence of a selection agent. In another embodiment step c) of the method is selecting a cell propagating in step b) and expressing the selectable marker encoded by said second nucleic acid. In the first embodiment is the transfected cell cultivated in the presence of a selection agent that inhibits the propagation of cells not transfected or not sufficiently expressing the second nucleic acid encoding the selectable marker. In the second embodiment is the transfected cell cultivated in the absence of a selection agent and selection is by the detection of the expression of the selectable marker, e.g. by FACS or sight inspection.

Selection of cells can be performed in a single step or in multiple steps. In a single/multiple step procedure the first selection can be performed based e.g. on a threshold level of a selectable marker, such as e.g. dLNGFR or GFP. For example, for selection by flow cytometry (e.g. by FACS—Fluorescence Activated Cell Sorting) a fluorescence threshold level is set and cells with a fluorescence above this threshold level are selected. Alternatively cells within the top 1-15% (i.e. the 15% of the cells with the most intense detectable label), or top 1-10%, or top 1-5%, or top 5-10% of fluorescence intensity of the sample population can be collected. An alternative method for the selection of a cell is immunological binding, e.g. to magnetic beads coated with Protein A or specific immunoglobulins. The selected panel of cells may be taken as basic population for a further selection step, e.g. by single cell seeding, cultivation and ELISA analysis (Enzyme-linked Immunosorbent Assay), or by limited dilution cloning, or by expanding by cultivation for several days and a further FACS selection, or by a further FACS selection with a higher threshold level, which can for example be based on the fluorescence intensities detected in a preceding FACS selection, or by an immunoprecipitation method (see e.g. WO 2005/020924). Selecting a cell according to the invention can in one embodiment be performed by a method selected from flow cytometry, ELISA, immunoprecipitation, immunoaffinity column chromatography, magnetic bead immunoaffinity sorting, microscopy-based isolation methods, or immunological binding. In another embodiment selecting a cell according to the invention can be performed by a method selected from flow cytometry, ELISA, immunoprecipitation, immunoaffinity column chromatography, magnetic bead immunoaffinity sorting, microscopy-based isolation methods, or immunological binding, followed by a method selected from single cell seeding and cultivation, limited dilution, or expanding by cultivation, followed by a method selected from FACS, immunoprecipitation, immunoaffinity column chromatography, magnetic bead immunoaffinity sorting, microscopy-based isolation methods, or ELISA.

The final aspect of the current invention is a method for the expression of a heterologous polypeptide in a cell by operably linking a promoter according to the invention to a nucleic acid encoding said heterologous polypeptide. This method is suitable for the expression e.g. of large proteins with low solubilities or slow folding kinetics. The reduction of the amount or rate of expression of a heterologous polypeptide or of the transcription of a nucleic acid is advisable if the heterologous polypeptide or nucleic acid adversely affects the host cell or reduces the overall production yield of functionable, i.e. correctly folded, heterologous polypeptide. Therefore, one aspect of the current invention is the expression or production of a heterologous polypeptide with reduced fraction of not functionable, i.e. not correctly folded, polypeptide. If the heterologous polypeptide expressed in the host cell e.g. exceeds a certain size with respect to weight, or amino acid number, or number of subunits, or number of secondary modifications, it probably will be obtained after the cultivation of the host cell in a non-functionable, i.e. non-active or not correctly folded, form. One possibility to circumvent this problem is to reduce the amount, i.e. the rate, of the protein expression. As protein expression is regulated by the strength of the operably linked promoter, the promoters according to the invention are well suited therefore.

Therefore, the current invention comprises a method for the expression or production of a heterologous polypeptide with reduced fraction of not functionable polypeptide wherein the method comprises the following steps in this order:

a) transfecting a mammalian cell with a nucleic acid comprising an expression cassette comprising a promoter of SEQ ID NO: 02, or SEQ ID NO: 03, or SEQ ID NO: 04, or SEQ ID NO: 06 operably linked to a nucleic acid encoding a heterologous polypeptide,

b) selecting a cell transfected in step a),

c) cultivating the selected cell under conditions suitable for the expression of the heterologous polypeptide,

d) recovering the heterologous polypeptide from the cell or the cultivation medium.

In one embodiment of this aspect of the current invention the mammalian cell is a CHO cell, a BHK cell, a HEK cell, a Sp2/0 cell, or a PER.C6® cell. In one embodiment of this method the promoter is of SEQ ID NO: 03 or SEQ ID NO: 04. In another embodiment has the promoter the SEQ ID NO: 04. In a further embodiment is the nucleic acid encoding a heterologous polypeptide encoding an immunoglobulin, or an immunoglobulin-fragment, or an immunoglobulin-conjugate. In still another embodiment comprises the nucleic acid a second expression cassette encoding a selectable marker.

The following examples, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

DESCRIPTION OF THE FIGURES

FIG. **1** Plasmid map of plasmid 5500.

FIG. **2** Plasmid map of plasmid 5501.

FIG. **3** Plasmid map of plasmid 4703.

FIG. **4** Plasmid map of plasmid 4712.

FIG. **5** Plasmid map of plasmid 4713.

FIG. **6** FACS analysis of dLNGFR-expression of HEK293EBNA-cells transfected with

a) an expression cassette of SEQ ID NO: 05 operably linked to SEQ ID NO: 07,

b) an expression cassette of SEQ ID NO: 04 operably linked to SEQ ID NO: 07,

c) an expression cassette of SEQ ID NO: 06 operably linked to SEQ ID NO: 07.

FIG. **7** FACS analysis of GFP-expression of HEK293EBNA-cells transfected with

a) an expression cassette of SEQ ID NO: 05 operably linked to SEQ ID NO: 07,

b) an expression cassette of SEQ ID NO: 03 operably linked to SEQ ID NO: 07,

c) an expression cassette of SEQ ID NO: 06 operably linked to SEQ ID NO: 07.

EXAMPLE 1

Construction of Nucleic Acid of SEQ ID NO: 02

The 5' shortened SV40 promoter of SEQ ID NO: 02 was obtained via a PCR reaction with the full length SV40 promoter as template operably linked to a nucleic acid encoding dLNGFR (plasmid 4788). The PCR mixture was: 1×PWO buffer (Roche Molecular Biochemicals, Mannheim, Germany) supplemented with 2 mM $MgSO_4$, 200 μM dNTPs PCR Nucleotide Mix (Roche Molecular Biochemicals, Mannheim, Germany), 1 μM forward primer of SEQ ID NO: 08, 1 μM reverse primer of SEQ ID NO: 13, 50 ng template-DNA of plasmid 4788, 2.5 U PWO-DNA polymerase (PWO=*Pyrococcus woesei*; Roche Molecular Biochemicals, Mannheim, Germany), ad 100 μL with doubly distilled ultrapure water. The PCR conditions were: 1 min at 94° C., 1 cycle; 0.5 min at 94° C., 25 cycles; 0.5 min at 55° C., 25 cycles; 1 min at 72° C., 25 cycles; 5 min at 72° C., 1 cycle.

EXAMPLE 2

Construction of Nucleic Acid of SEQ ID NO: 03

The 5' shortened SV40 promoter variant of SEQ ID NO: 03 was obtained via a PCR reaction with the full length SV40 promoter as template operably linked to a nucleic acid encoding dLNGFR from plasmid 4788. The PCR mixture was: 1×PWO buffer supplemented with 2 mM $MgSO_4$, 200 μM dNTPs PCR Nucleotide Mix, 1 μM forward primer of SEQ ID NO: 09, 1 μM reverse primer of SEQ ID NO: 13, 50 ng template-DNA of plasmid 4788, 2.5 U PWO-DNA polymerase, ad 100 μL with doubly distilled ultrapure water. The PCR conditions were: 1 min at 94° C., 1 cycle; 0.5 min at 94° C., 25 cycles; 0.5 min at 55° C., 25 cycles; 1 min at 72° C., 25 cycles; 5 min at 72° C., 1 cycle.

EXAMPLE 3

Construction of Nucleic Acid of SEQ ID NO: 04

The 5' shortened SV40 promoter variant of SEQ ID NO: 04 was obtained via a PCR reaction with the full length SV40 promoter as template operably linked to a nucleic acid encoding dLNGFR (plasmid 4788). The PCR mixture was: 1×PWO buffer supplemented with 2 mM $MgSO_4$, 200 μM dNTPs PCR Nucleotide Mix, 1 μM forward primer of SEQ ID NO: 10, 1 μM reverse primer of SEQ ID NO: 13, 50 ng template-DNA of plasmid 4788, 2.5 U PWO-DNA polymerase, ad 100 μL, with doubly distilled ultrapure water. The PCR conditions were: 1 min at 94° C., 1 cycle; 0.5 min at 94° C., 25 cycles; 0.5 min at 55° C., 25 cycles; 1 min at 72° C., 25 cycles; 5 min at 72° C., 1 cycle.

EXAMPLE 4

Construction of Further Promoters

Further 5' shortened SV40 promoter variants were produced via a PCR reaction with the full length SV40 promoter as template operably linked to a nucleic acid encoding dLNGFR. The PCR mixture was: 1×PWO buffer supplemented with 2 mM $MgSO_4$, 200 μM dNTPs PCR Nucleotide Mix, 1 μM forward primer of SEQ ID NO: 11 (yielding SEQ ID NO: 06) or SEQ ID NO: 12, 1 μM reverse primer of SEQ ID NO: 13, 50 ng template-DNA of Plasmid 4788, 2.5 U PWO-DNA polymerase, ad 100 μL with bidistilled ultrapure water. The PCR conditions were: 1 min at 94° C., 1 cycle; 0.5 min at 94° C., 25 cycles; 0.5 min at 55° C., 25 cycles; 1 min at 72° C., 25 cycles; 5 min at 72° C., 1 cycle.

EXAMPLE 5

Expression of dLNGFR Operably Linked to SEQ ID NO: 02, 03, 04, and 06

The primer with which the nucleic acids of SEQ ID NO: 02, 03, 04, and 06 were obtained contained restriction sites of the restriction endonucleases SalI and EcoRI. Using these

restriction sites/restriction endonucleases these nucleic acids operably linked to a nucleic acid encoding dLNGFR (for LNGFR (low affinity nerve growth factor) see e.g. Philipps, K., et al., Nat. Med. 2 (1996) 1154-1156; or Machl, A. W., et al., Cytometry 29 (1997) 371-374) have been ligated into the plasmid 4736-pUC-DHFR, which has been linearized using the restriction sites SalI and PvuII. The resulting plasmids are:

5500-pUC-DHFR_dLNGFR_wildtypeSV40 (plasmid map in FIG. **1**),

5501-pUC-DHFR_dLNGFR_Shortening_2 (plasmid map in FIG. **2**),

5502-pUC-DHFR_dLNGFR_Shortening_3,

5503-pUC-DHFR_dLNGFR_Shortening_4,

5504-pUC-DHFR_dLNGFR_Shortening_6.

HEK 293 EBNA cells have been transfected with these plasmids and the encoded polypeptide was transiently expressed. After 48 h the expression of dLNGFR has been verified via FACS. For the determination of the promoter strength (expression strength) of the different promoters the expressed surface marker dLNGFR was fluorescence marked via an anti-dLNGFR antibody.

For each determination approximately 0.5×10^6 to 1.0×10^6 cells have been detached by the addition of 1 ml Accutase® per 6 wells (GIBCO Invitrogen, Karlsruhe, Germany). The detached cells were transferred in a vial and washed once with RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum. Afterwards the cells were precipitated by centrifugation (1,500 rpm, 5 min.) and the supernatant was discarded. All following steps were performed at 0 to 2° C. on or in an ice bath. The cell pellet was resuspended in 100 μl of a solution containing the anti-dLNGFR antibody at 30 μg/ml. After an incubation period of 30 minutes the samples were diluted by the addition of 2 ml of ice-cold RPMI 1640 medium with subsequent precipitation by centrifugation. The pellet was resuspended in 100 μL of a secondary antibody solution, a goat anti-mouse-IgG antibody conjugated to Phycoerythrin (Caltag Laboratories, Burlingame, Calif., USA), at a concentration of 20 μg/ml. The sample was incubated in the dark for 30 min. on ice. After a washing and centrifugation step the sample was resuspended in 500 μl medium and stored in the dark on ice until the measurement. The FACS analysis was evaluated using the FACSCalibur software (Cell Quest Pro). The results are shown in FIG. **6**.

Results of the FACS analysis:

5500-pUC-DHFR_dLNGFR_wildtypeSV40 (FIG. **6***a*)):

Marker	Left, Right	Events	% Gated	% Total	Mean	Median
All	1, 9910	8067	100.00	80.67	567.61	128.64
M1	1, 40	2134	26.45	21.34	21.84	21.29
M2	40, 9910	5956	73.83	59.56	761.11	345.99

5501-pUC-DHFR_dLNGFR_Shortening_2:

Marker	Left, Right	Events	% Gated	% Total	Mean	Median
All	1, 9910	7377	100.00	73.77	564.33	168.49
M1	1, 40	1365	18.50	13.65	24.69	25.48
M2	40, 9910	6027	81.70	60.27	685.24	291.64

5502-pUC-DHFR_dLNGFR_Shortening_3:

Marker	Left, Right	Events	% Gated	% Total	Mean	Median
All	1, 9910	7643	100.00	76.43	582.85	129.80
M1	1, 40	1959	25.63	19.59	22.68	22.88
M2	40, 9910	5708	74.68	57.08	772.82	339.82

5503-pUC-DHFR_dLNGFR_Shortening_4 (FIG. **6***b*)):

Marker	Left, Right	Events	% Gated	% Total	Mean	Median
All	1, 9910	7440	100.00	74.40	436.61	69.78
M1	1, 40	2603	34.99	26.03	20.87	19.99
M2	40, 9910	4852	65.22	48.52	658.42	250.29

5504-pUC-DHFR_dLNGFR_Shortening_6 (FIG. **6***c*)):

Marker	Left, Right	Events	% Gated	% Total	Mean	Median
All	1, 9910	8404	100.00	84.04	31.67	11.65
M1	1, 40	6685	79.55	66.85	12.05	9.14
M2	40, 9910	1732	20.61	17.32	107.45	74.32

It can be seen that the mean fluorescence intensity of the labeled dLNGFR expressed from plasmid 5504 shows a significant reduction of expression with about 85% reduction.

EXAMPLE 6

Expression of GFP Operably Linked to SEQ ID NO: 02, 03, 04, and 06

The primer with which the nucleic acids of SEQ ID NO: 02, 03, 04, and 06 were obtained contained restriction sites of the restriction endonucleases SalI and EcoRI. Using these restriction sites/restriction endonucleases these nucleic acids operably linked to a nucleic acid encoding GFP (SEQ ID NO: 07) have been ligated into the plasmid 4703-pUC-OriP (FIG. **3**), which has been linearized using the restriction sites SalI and PvuII. The resulting plasmids were:

4712-pUC-Hyg_GFP_wildtypeSV40 (plasmid map in FIG. **4**),

4713-pUC-Hyg_GFP_Shortening_2 (plasmid map in FIG. **5**),

4714-pUC-Hyg_GFP_Shortening_3,

4715-pUC-Hyg_GFP_Shortening_4,

4716-pUC-Hyg_GFP_Shortening_6.

For each determination approximately 5×10^5 to 1×10^6 cells have been detached by the addition of 1 ml Accutase® per 6 wells (GIBCO Invitrogen, Karlsruhe, Germany). The detached cells were transferred in a vial and resuspended in 3 ml RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum. Afterwards the cells were precipitated by centrifugation (1,500 rpm, 5 min.) and the supernatant was discarded. The cell pellet was resuspended in 500 μl medium. For the differentiation of living and dead cells 1 μl propidium iodide was added. The cells were resuspended shortly prior to the FACS measurement. The FACS analysis was evaluated using the FACSCalibur software (Cell Quest Pro). The results are shown in FIG. **7**.

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Results of the FACS Analysis:
4712-pUC-Hyg_GFP_wildtypeSV40 (FIG. 7*a*)):

Marker	Left, Right	Events	% Gated	% Total	Mean	Median
All	1, 9910	8390	100.00	33.90	385.26	17.15
M1	1, 16	4162	49.61	41.62	5.92	4.91
M2	16, 9910	4240	50.54	42.40	756.58	302.32

4713-pUC-Hyg_GFP_Shortening_2:

Marker	Left, Right	Events	% Gated	% Total	Mean	Median
All	1, 9910	8576	100.00	85.76	514.24	45.73
M1	1, 16	3635	42.39	36.35	5.72	4.61
M2	16, 9910	4948	57.70	49.48	887.12	392.42

4714-pUC-Hyg_GFP_Shortening_3 (FIG. 7*b*):

Marker	Left, Right	Events	% Gated	% Total	Mean	Median
All	1, 9910	8538	100.00	85.38	215.22	15.96
M1	1, 16	4289	50.23	42.89	5.44	4.26
M2	16, 9910	4258	49.87	42.58	426.11	203.51

4715-pUC-Hyg_GFP_Shortening_4:

Marker	Left, Right	Events	% Gated	% Total	Mean	Median
All	1, 9910	8601	100.00	86.01	53.76	7.37
M1	1, 16	5606	65.18	56.06	5.59	4.41
M2	16, 9910	3012	35.02	30.12	143.20	73.65

4716-pUC-Hyg_GFP_Shortening_6 (FIG. 7*c*)):

Marker	Left, Right	Events	% Gated	% Total	Mean	Median
All	1, 9910	7622	100.00	76.22	4.09	3.52
M1	1, 16	7614	99.90	76.14	4.07	3.52
M2	16, 9910	8	0.10	0.08	22.46	18.85

It can be seen that the mean fluorescence intensity of the GFP expressed from plasmid 4714 or plasmid 4715 shows a significant reduction of expression with about 50% and 75% reduction, respectively. With plasmid 4716 no detectable expression of GFP was found.

SEQUENCE LISTING

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tgcatctcaa ttagtcagca accatagtcc cgcccctaac tccgcccatc ccgcccctaa 180

ctccgcccag ttccgcccat tctccgcccc atggctgact aatttttttt atttatgcag 240

aggccgaggc cgcctctgcc tctgagctat tccagaagta gtgaggaggc ttttttggag 300

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cccgggagct tgtatatcca ttttcg 146

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aaacttaccc ttaaatttat ttgcactact ggaaagctac ctgttccatg gccaacactt    180
gtcactactt tctcttatgg tgttcaatgc ttttcaagat acccagatca tatgaaacag    240
catgactttt tcaagagtgc catgcccgaa ggttatgtac aggaaagaac tatattttac    300
aaagatgacg ggaactacaa atcacgtgct gaagtcaagt ttgaaggtga taccctcgtt    360
aatagaattg agttaaaagg tattgatttt aaagaagatg gaaacattct tggacacaaa    420
atggaataca actataactc acacaatgta tacatcatgg cagacaaaca aaagaatgga    480
atcaaagtta acttcaaaat tagacacaac attgaagatg gaagcgttca actagcagac    540
cattatcaac aaaatactcc aattggcgat ggccctgtcc ttttaccaga caaccattac    600
ctgtccacac aatctgccct ttccaaagat cccaacgaaa agagagatca catgatcctt    660
cttgagtttg taacagctgc tgggattaca catggcatgg atgaactata caaataa       717
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actttccaca cc                                                        72
```

The invention claimed is:

1. A method for the selection of a cell expressing a heterologous polypeptide to the cell expressing it comprising the following steps:

a) transfecting an isolated eukaryotic cell with a nucleic acid comprising

i) a first expression cassette comprising a nucleic acid encoding a heterologous polypeptide,

ii) a second expression cassette comprising a first nucleic acid comprising the sequence of SEQ ID NO: 04 and a second nucleic acid encoding a selectable marker selected from the group consisting of hygromycin phosphotransferase, neomycin and G418 aminoglycoside phosphotransferase, dLNGFR and GFP, whereby said first and second nucleic acid are operably linked,

b) cultivating said transfected cell under conditions suitable for the growth of non-transfected cell; and

c) cultivating said cells under selective culture conditions;

d) selecting a cell propagating in step b) and under selective culture conditions in step c).

2. The method of claim **1**, wherein step d) of said method is selecting a cell propagating in step b) and expressing the selectable marker encoded by said second nucleic acid.

3. A method for the expression of a heterologous polypeptide to the cell expressing it, comprising the following steps:

a) transfecting an isolated eukaryotic cell with a nucleic acid comprising an expression cassette comprising a first nucleic acid having the sequence of SEQ ID NO: 04 operably linked to a second nucleic acid encoding a heterologous polypeptide,

b) selecting a cell transfected in step a),

c) cultivating the selected cell of step b) under conditions suitable for the expression of said heterologous polypeptide; and

d) recovering the heterologous polypeptide from the cell or the cultivation medium.

4. The method of claim **3**, wherein the nucleic acid comprises a second expression cassette encoding an aminoglycoside phosphotransferase selected from the group consisting of hygromycin phosphotransferase, neomycin and G418 aminoglycoside phosphotransferase.

5. The method of claim **1**, wherein said eukaryotic cell is a mammalian cell.

6. The method of claim **5**, wherein said mammalian cell is a CHO cell, a BHK cell, a HEK cell or, a Sp2/0 cell.

7. The method of claim **6**, wherein said mammalian cell is a CHO cell or a HEK cell.

8. The method of claim **1**, wherein said heterologous polypeptide is an immunoglobulin, or an immunoglobulin-fragment, or an immunoglobulin-conjugate.

9. The method of claim **3**, wherein said first nucleic acid is a nucleic acid having the sequence of SEQ ID NO: 04 and has a promoter strength of 20% or less of the SV40 promoter of SEQ ID NO: 05 when operably linked to the nucleic acid of SEQ ID NO: 07.

* * * * *

EXHIBIT FF

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US009428766B2

(12) **United States Patent**
Goepfert et al.

(10) **Patent No.: US 9,428,766 B2**
(45) **Date of Patent: *Aug. 30, 2016**

(54) **PROTEIN EXPRESSION FROM MULTIPLE NUCLEIC ACIDS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **14/293,447**

(22) Filed: **Jun. 2, 2014**

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Related U.S. Application Data

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(30) **Foreign Application Priority Data**

Oct. 12, 2007 (EP) 07019999

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C07K 16/28	(2006.01)
C12P 21/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C12N 15/85*** (2013.01); ***C07K 16/18*** (2013.01); ***C07K 16/28*** (2013.01); ***C07K 16/2812*** (2013.01); ***C07K 16/2854*** (2013.01); ***C07K 16/2866*** (2013.01); ***C07K 16/2896*** (2013.01); ***C12P 21/00*** (2013.01); *C07K 2317/14* (2013.01); *C07K 2317/21* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,420,019	A	5/1995	Theofan et al.
5,550,036	A	8/1996	Grinnell
5,639,275	A	6/1997	Baetge et al.
5,852,175	A	12/1998	Cummings et al.
2003/0096341	A1	5/2003	Mueller et al.
2005/0169925	A1	8/2005	Bardroff et al.
2005/0226876	A1	10/2005	Graus et al.

FOREIGN PATENT DOCUMENTS

EP	0319206		6/1989
EP	0921194		6/1996
EP	1010758		6/2000
WO	8900605		1/1989
WO	89/10959	A1	11/1989
WO	93/01296	A1	1/1993
WO	94/25067	A1	11/1994
WO	95/17513	A1	6/1995
WO	99/47647	A1	9/1999
WO	0028066		5/2000
WO	03/046187		6/2003
WO	03070760		8/2003
WO	2004/083247	A1	9/2004
WO	2004/087758	A2	10/2004

(Continued)

OTHER PUBLICATIONS

Jung, H. et al., Detection and treatment of mycoplasma contaminiation in cultured cells , Apr. 2003, CHang Gung Med. J., vol. 26, pp. 250-258.*

The English translation of the Chinese Office Action, issued on Mar. 7, 2013, in the corresponding Chinese application No. 200880110216.6.

Summons to Oral Proceeding by the EPO, mailed on Oct. 8, 2014, in related European Patent No. 1453966.

(Continued)

Primary Examiner — Michael Burkhart

(74) *Attorney, Agent, or Firm* — Jones Day

(57) **ABSTRACT**

The current invention reports a method for the recombinant production of a secreted heterologous immunoglobulin in a CHO cell comprising the following steps: i) providing a CHO cell, which is adapted to growth in suspension culture, adapted to growth in serum-free medium, mycoplasma free, and virus free, ii) providing a vector comprising a prokaryotic origin of replication, a first nucleic acid conferring resistance to a prokaryotic selection agent, a second nucleic acid encoding the heavy chain of said heterologous immunoglobulin, a third nucleic acid encoding the light chain of said heterologous immunoglobulin, a fourth nucleic acid conferring resistance to a eukaryotic selection agent, iii) transfecting said CHO cell, wherein said transfecting comprises a) transfecting said CHO cell with said vector comprising a fourth nucleic acid conferring resistance to a first eukaryotic selection agent, b) selecting a CHO cell by growth in cultivation medium containing said first eukaryotic selection agent, c) transfecting said selected CHO cell with said vector comprising a fourth nucleic acid conferring resistance to a second eukaryotic selection agent different to said first eukaryotic selection agent, d) selecting a CHO cell by selected growth in cultivation medium containing said first and said second eukaryotic selection agent, iv) cultivating said transfected CHO cell in a medium in the presence of said first and second eukaryotic selection agent, under conditions suitable for the expression of said second, and third nucleic acid, and v) recovering said secreted heterologous immunoglobulin from the cultivation medium.

1 Claim, 9 Drawing Sheets

(56) **References Cited**

FOREIGN PATENT DOCUMENTS

WO	2005100402		10/2005
WO	2006/072564	A1	7/2006
WO	2007/109052	A2	9/2007
WO	2007/113172	A2	10/2007
WO	2009/012944		1/2009

OTHER PUBLICATIONS

Opposition against EP 1453966 by Strawman Ltd. on Apr. 11, 2013.
Opposition against EP 1453966 by Boehringer Ingelheim on Apr. 10, 2013.
D4: Plasmid: pSV2-dhfr, Mar. 16, 2013.
D5: ATCC product sheet-pSV2-dhfr, Feb. 22, 2013.
D9: Lin et al., "Cloning and expression of the human erythropoietin gene," Proc Natl Acad Sci U S A. Nov. 1985; 82 (22): 7580-7584.
D11: Chen et al., "Production of Recombinant Proteins in Mammalian Cells," Current Protocols in Protein Science, Supplement 12, pp. 5.10.1-5.10.41, 1998.
D15: Kemball-Cook et al., "High-level production of human blood coagulation factors VII and XI using a new mammalian expression vector," Gene. Feb. 25, 1994;139(2):275-9.
D16: Sambrook et al., "Molecular Cloning: A Laboratory Manual," 2001, vol. 3.
D18: Plasmid: pSV2neo, Mar. 20, 2013.
D19: Mulligan et al., "Selection for animal cells that express the *Escherichia coli* gene coding for xanthine guanine phosphoribosyltransferase," Proc. Natl Acad. Sci. USA 78, 2072-2076, Apr. 1981.
D20: Uniprot entry P0A9M5, Xanthine-guanine phosphoribosyltransferase from *E. coli*, Jun. 21, 1986.
D21: Colosimo, A., et al., "Transfer and expression of foreign genes in mammalian cells," Biotechniques, 2000. 29(2): pp. 314-331.
D22: Karam et al., "Methods in Nucleic Acids Research," Chapter 14, pp. 283-305, 1991 by CRC Press, Inc.
D23: Commission decision granting the marketing authorization for the medicinal product for human use, "Ceprotein-Protein C." Jul. 16, 2001.
D24: Wang et al., "Treating acute stroke patients with intravenous tPA. The OSF stroke network experience," Stroke. Jan. 2000;31(1):77-81.
D30: Webpage from ATCC showing details of the pPA509 vector of D8, Feb. 9, 2015.
The Interlocutory Decision, issued on Jul. 6, 2015, in opposition proceedings in related European Patent No. 1453966.
D26: Product sheet of CRL-9096 cells, 2013.
D25: Declaration of independent expert witness, Dr. Holly Prentice, Feb. 6, 2015.
D29: Webpage from Amazon.com showing the publication date of D16 (Molecular Cloning: A Laboratory Manual (2001) Third Edition), Nov. 24, 2014.
Lee et al., "Development of a serum-free medium for the production of erythropoietin by suspension culture of recombinant Chinese hamster ovary cells using a statistical design," J. Biotechnol. 69(2-3):85-93 (1999).
Li et al., "A comparative study of different vector designs for the mammalian expression of recombinant IgG antibodies," J. Immunol. Methods. 318(1-2):113-124 (2007) (Epub Nov. 13, 2006).
Moretto et al., "Conformation-sensitive antibodies against alzheimer amyloid-beta by immunization with a thioredoxin-constrained B-cell epitope peptide," J. Biol. Chem. 282(15):11436-11445 (2007) (Epub Jan. 31, 2007).
Sakamoto et al., "Prevention of arterial reocclusion after thrombolysis with activated protein C. Comparison with heparin in a canine model of coronary artery thrombosis," Circulation 90(1):427-432 (1994).

* cited by examiner

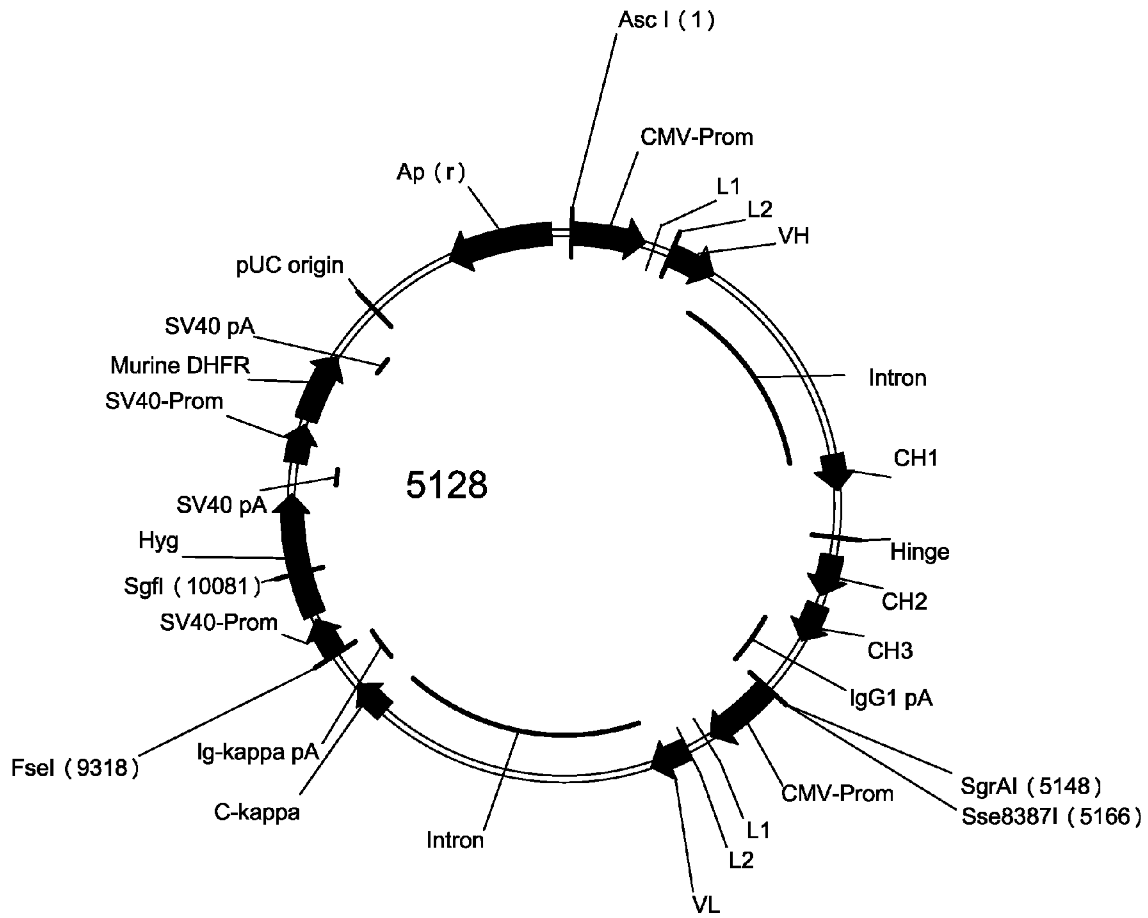


Fig. 1

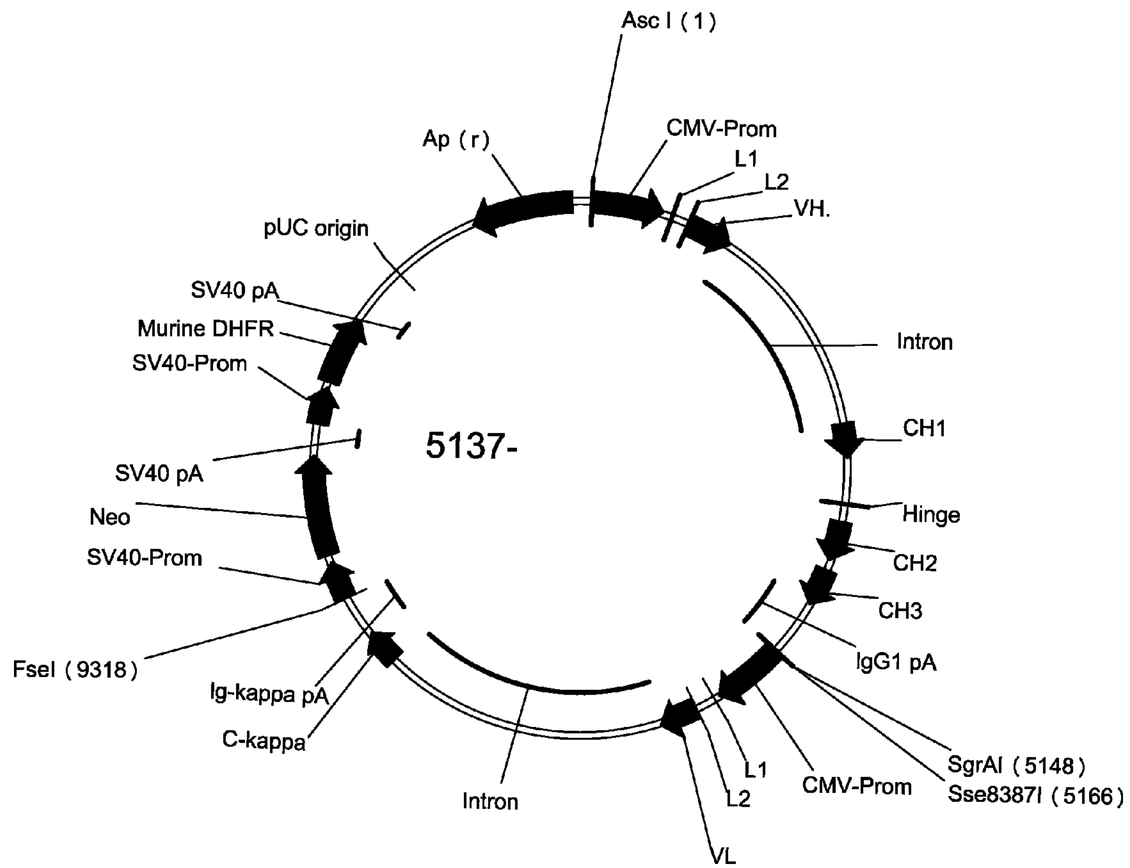


Fig. 2

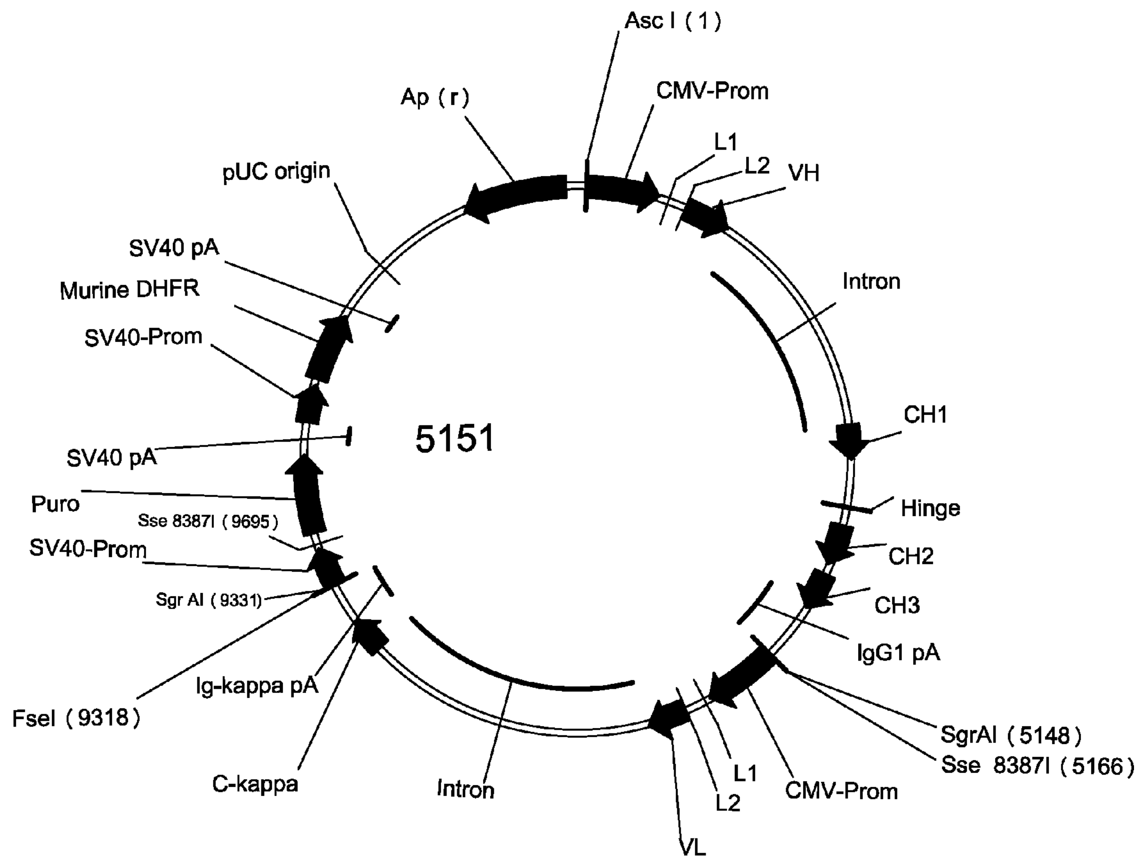


Fig. 3

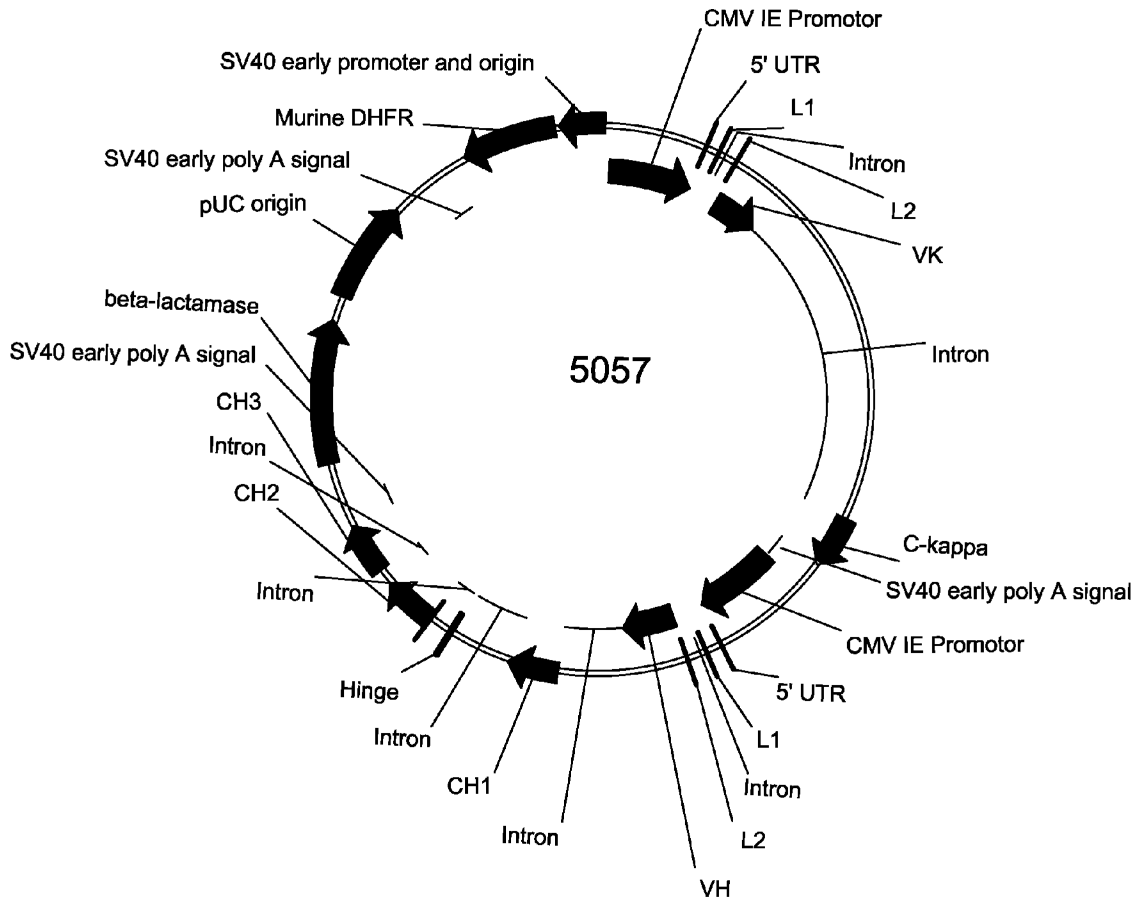


Fig. 4

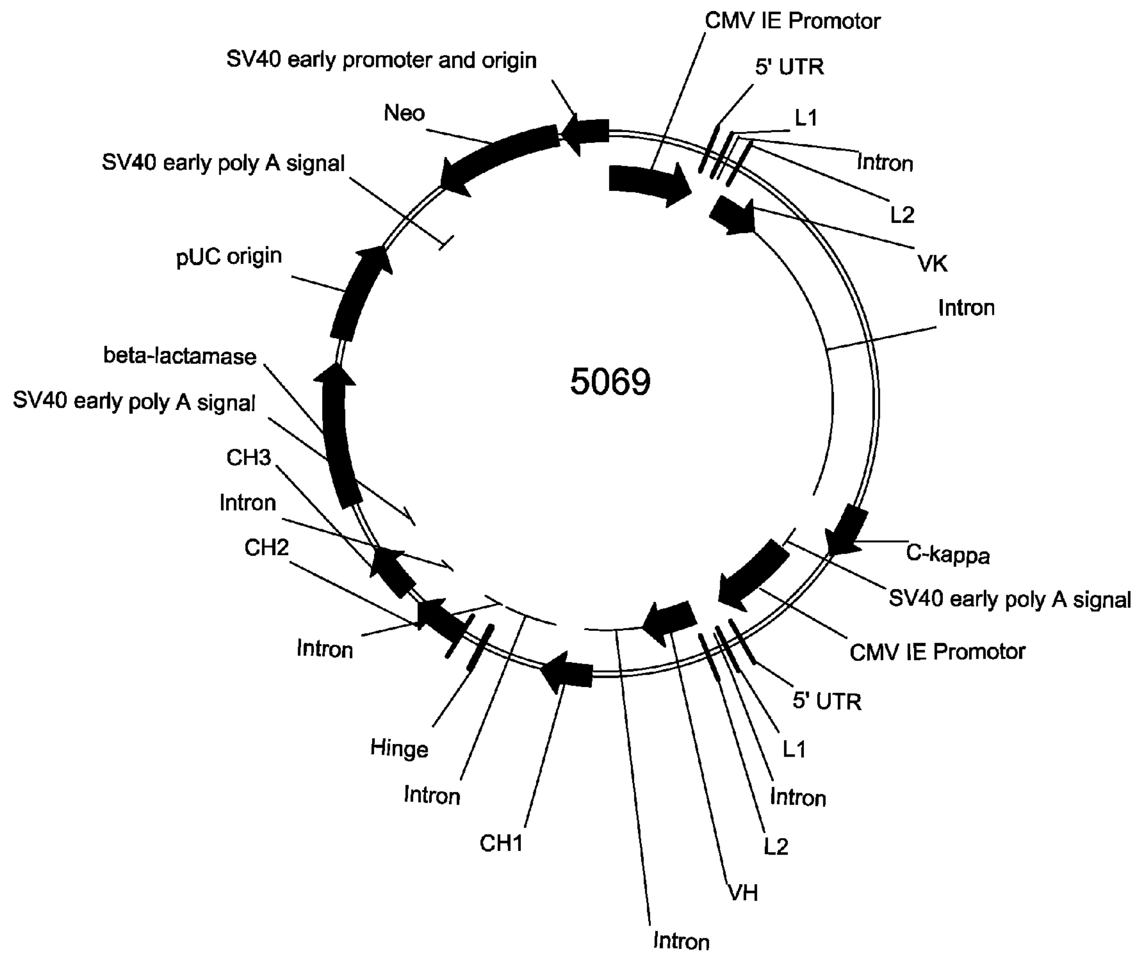
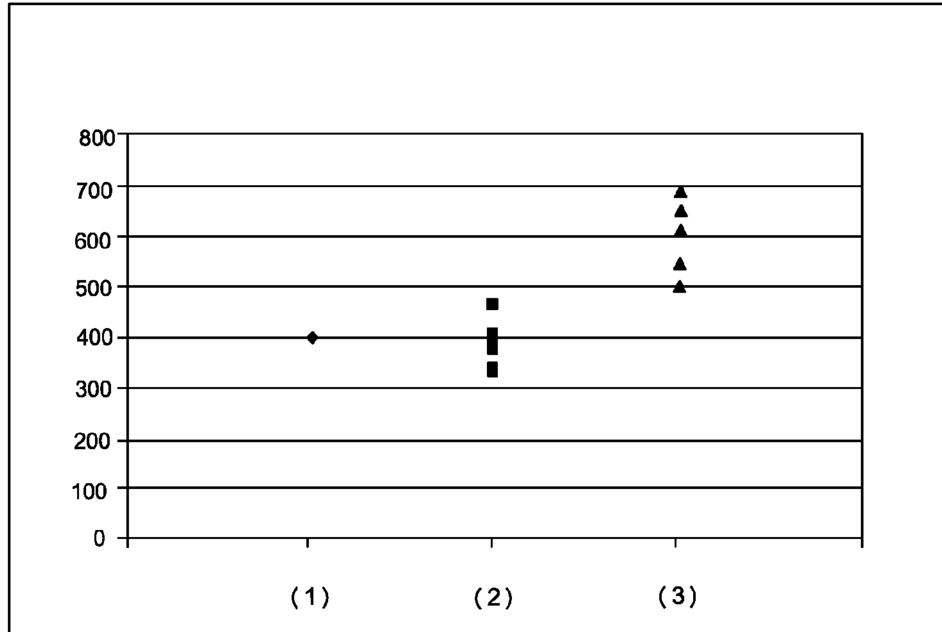


Fig. 5

(A)



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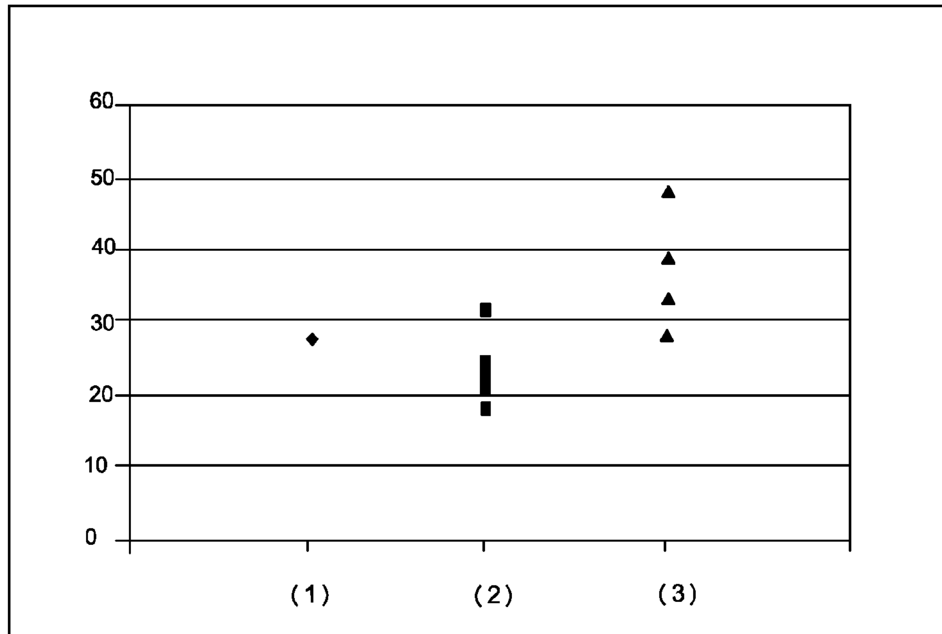

Fig. 6

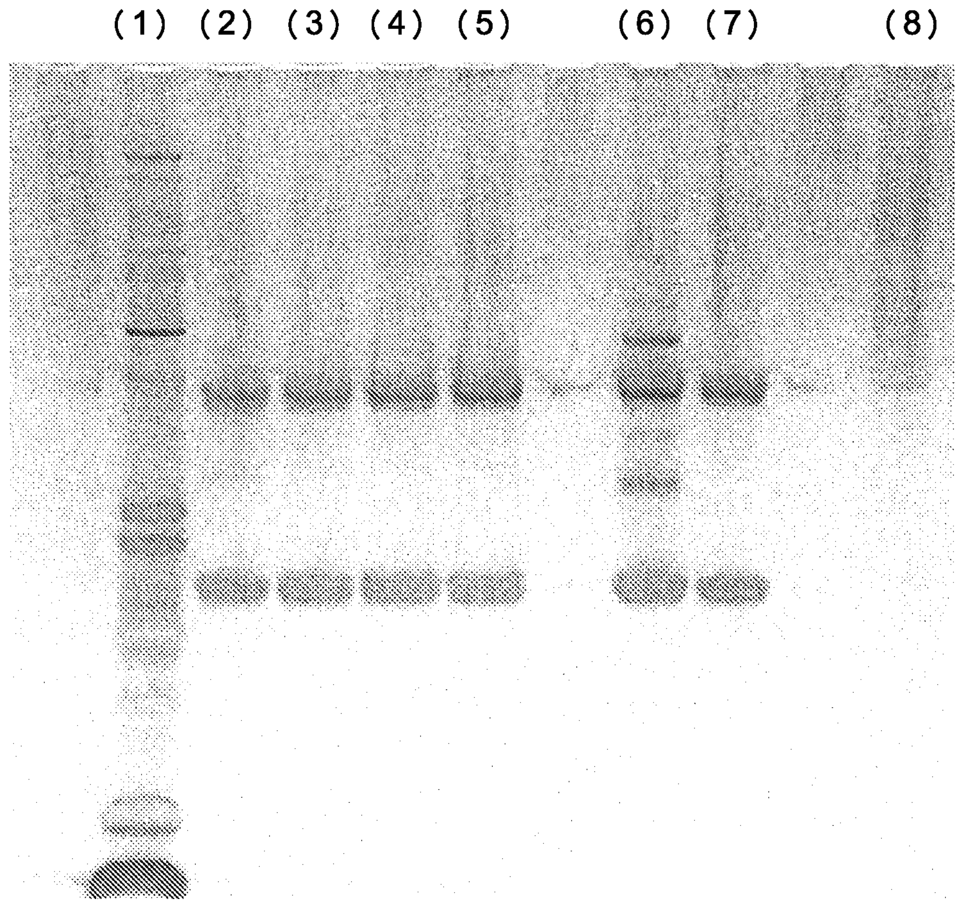

Fig. 7

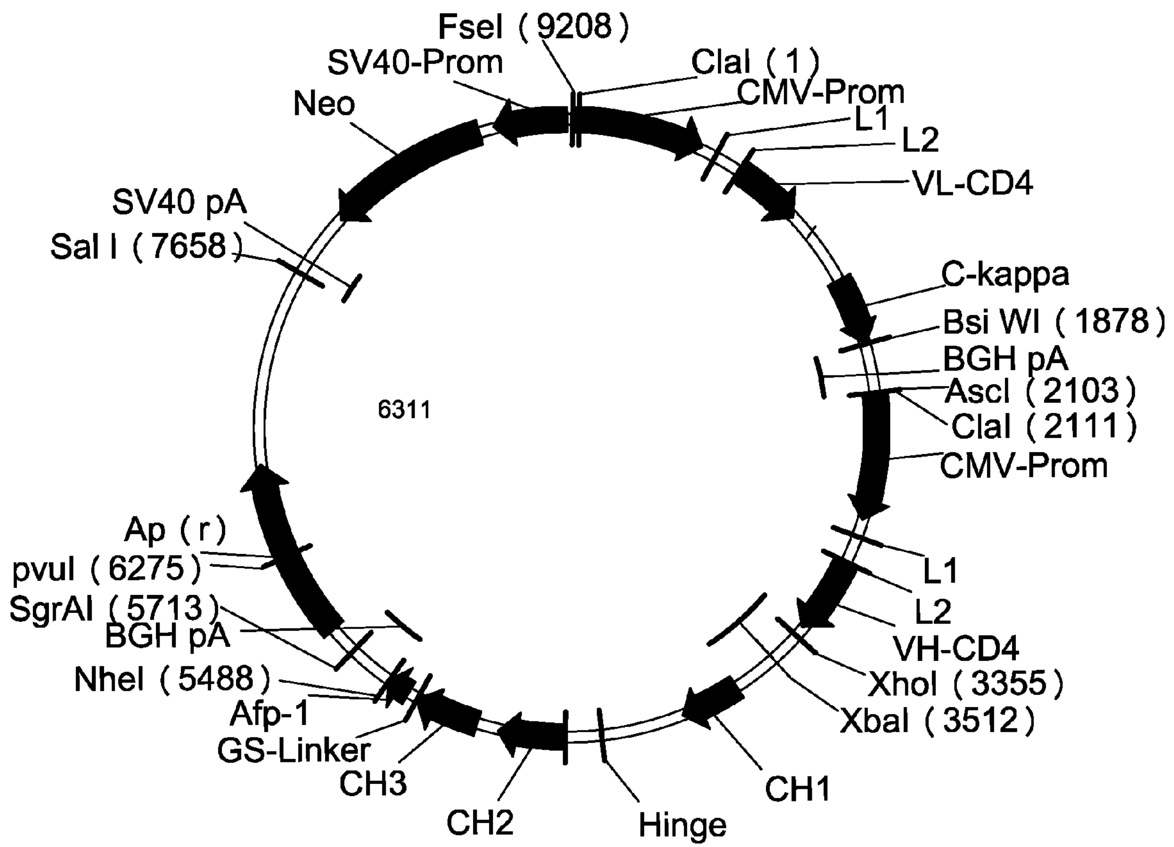

Fig. 8

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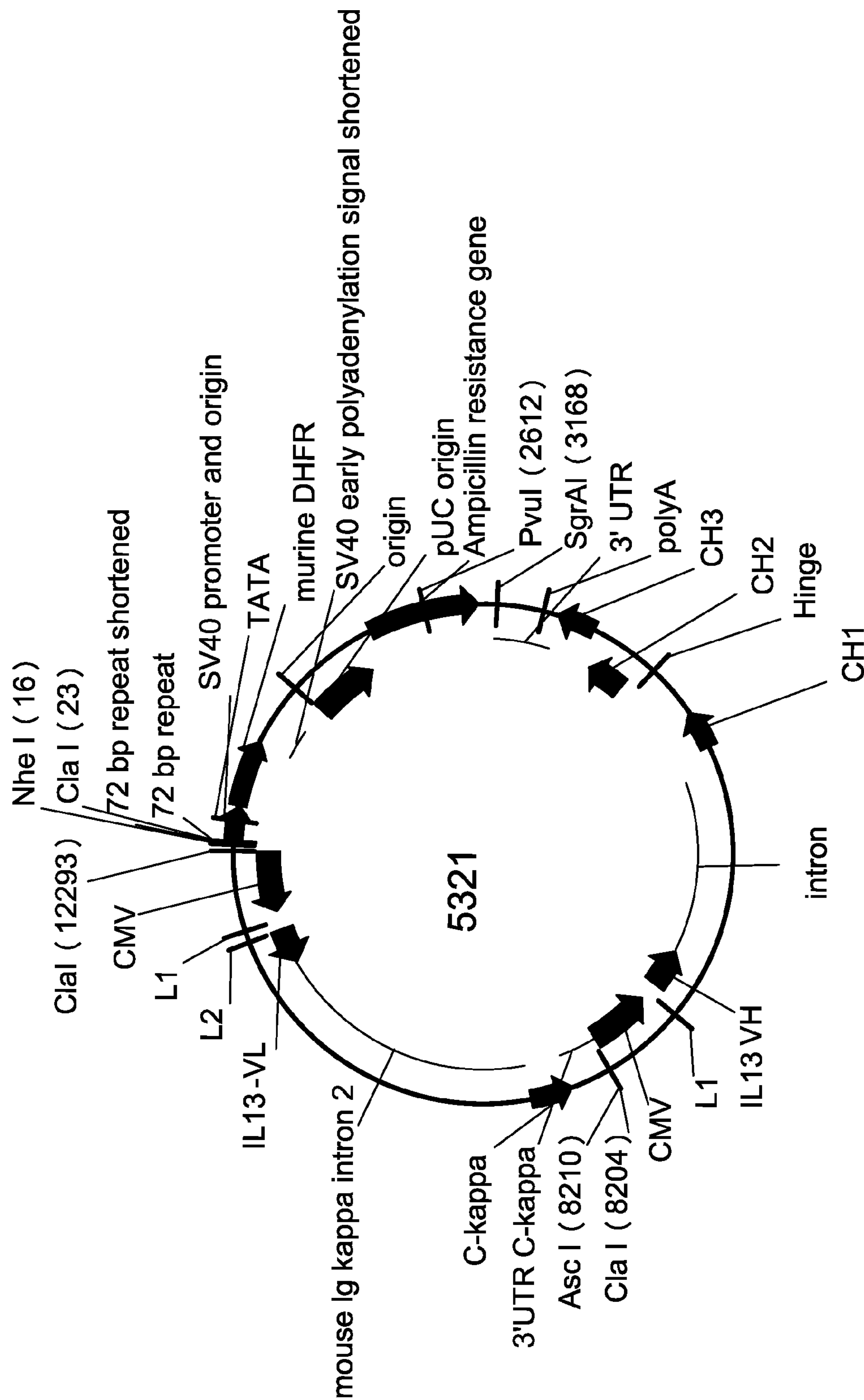

Fig. 9

PROTEIN EXPRESSION FROM MULTIPLE NUCLEIC ACIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to and is a continuation of pending U.S. application Ser. No. 12/681,781, filed Apr. 6, 2010, which in turn claims the benefit of International Application No. PCT/EP2008/008523, filed Oct. 9, 2008, which claim the benefit of European Patent Application No. 07019999.7 filed Oct. 12, 2007 both of which are hereby incorporated by reference in their entirety.

FILED OF THE INVENTION

The current invention is in the field of polypeptide production. More precisely it is reported the production of an immunoglobulin in a mammalian cell whereby the mammalian cell is transfected with different vectors each comprising an expression cassette for the immunoglobulin of interest.

BACKGROUND OF THE INVENTION

Expression systems for the production of recombinant polypeptides are well-known in the state of the art and are described by, e.g., Marino, M. H., Biopharm. 2 (1989) 18-33; Goeddel, D. V., et al., Methods Enzymol. 185 (1990) 3-7; Wurm, F., and Bernard, A., Curr. Opin. Biotechnol. 10 (1999) 156-159. Polypeptides for use in pharmaceutical applications are preferably produced in mammalian cells such as CHO cells, NS0 cells, SP2/0 cells, COS cells, HEK cells, BHK cells, PER.C6® cells, or the like. The essential elements of an expression plasmid are a prokaryotic plasmid propagation unit, for example for *E. coli*, comprising a prokaryotic origin of replication and a prokaryotic selection marker, an eukaryotic selection marker, and one or more expression cassettes for the expression of the structural gene(s) of interest each comprising a promoter, a structural gene, and a transcription terminator including a polyadenylation signal. For transient expression in mammalian cells a mammalian origin of replication, such as the SV40 Ori or OriP, can be included. As promoter a constitutive or inducible promoter can be selected. For optimized transcription a Kozak sequence may be included in the 5' untranslated region. For mRNA processing, in particular mRNA splicing and transcription termination, mRNA splicing signals, depending on the organization of the structural gene (exon/intron organization), may be included as well as a polyadenylation signal.

Expression of a gene is performed either as transient or as permanent expression. The polypeptide(s) of interest are in general secreted polypeptides and therefore contain an N-terminal extension (also known as the signal sequence) which is necessary for the transport/secretion of the polypeptide through the cell into the extracellular medium. In general, the signal sequence can be derived from any gene encoding a secreted polypeptide. If a heterologous signal sequence is used, it preferably is one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For secretion in yeast for example the native signal sequence of a heterologous gene to be expressed may be substituted by a homologous yeast signal sequence derived from a secreted gene, such as the yeast invertase signal sequence, alpha-factor leader (including *Saccharomyces, Kluyveromyces, Pichia*, and *Hansenula* α-factor leaders, the second described in U.S. Pat. No. 5,010,182), acid phosphatase signal sequence, or the *C. albicans* glucoamylase signal sequence (EP 0 362 179). In mammalian cell expression the native signal sequence of the protein of interest is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from secreted polypeptides of the same or related species, e.g. for immunoglobulins from human or murine origin, as well as viral secretory signal sequences, for example, the herpes simplex glycoprotein D signal sequence. The DNA fragment encoding for such a presegment is ligated in frame to the DNA fragment encoding a polypeptide of interest.

Today CHO cells are widely used for the expression of pharmaceutical polypeptides, either at small scale in the laboratory or at large scale in production processes. Due to their wide distribution and use the characteristic properties and the genetic background of CHO cells is well known. Therefore, CHO cells are approved by regulatory authorities for the production of therapeutic proteins for application to human beings.

In EP 0 569 678 are reported double transfectants of MHC genes as cellular vaccines for immunoprevention of tumor metastasis. WO 97/08342 reports an improved method for measuring the activity of a promoter sequence in a mammalian cell using a reporter gene. The use of anti-RhoA and anti-RhoC siRNAs in order to inhibit specifically RhoA or RhoC synthesis is reported in WO 2005/113770. A method for the recombinant production or expression of eukaryotic alkaline phosphatase mutant in yeast cells is reported in U.S. Pat. No. 7,202,072. WO 2001/038557 reports a method of screening multiply transformed cells using bicistronic expression of fluorescent proteins. A method for producing recombinant eukaryotic cell lines expressing multiple proteins or RNAs of interest is reported in WO 1999/47647. Systems, including methods, compositions, and kits, for transfection of cells with transfection materials using coded carriers are reported in WO 2003/076588. In U.S. Pat. No. 5,089,397 is reported an expression system for recombinant production of a desired protein comprising CHO cells transformed with a DNA sequence having the desired protein coding sequence under the control of the human metallothionein-II promoter. A method for producing recombinant proteins is reported in US 2003/0040047. Lamango et al. (Lamango, N. S., et al., Arch. Biochem. Biophys. 330 (1996) 238-250) report the dependency of the production of prohormone convertase 2 from the presence of the neuroendocrine polypeptide 7B2. The transfection of a BPV-1-based expression vector into cells harboring unintegrated replicating BPV-1 genomes is reported by Waldenstroem, M., et al., Gene 120 (1992) 175-181. U.S. Pat. No. 4,912,038 reports methods and vectors for obtaining canine and human 32K alveolar surfactant protein. In WO 89/10959 are reported recombinant DNA techniques and the expression of mammalian polypeptides in genetically engineered eukaryotic cells. A repeated co-transfer of an expression vector for human growth hormone and an expression vector for a selection marker gene is reported in DD 287531.

SUMMARY OF THE INVENTION

A first aspect of the current invention is a method for the recombinant production of a heterologous immunoglobulin which is secreted to the cultivation medium in a CHO cell comprising:

a) providing a CHO cell, which is
adapted to growth in suspension culture,

adapted to growth in serum-free medium,
mycoplasm free, and
optional virus free,

b) providing a nucleic acid comprising
a prokaryotic origin of replication,
a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,
a second nucleic acid sequence encoding the heavy chain of said heterologous immunoglobulin, and/or a third nucleic acid sequence encoding the light chain of said heterologous immunoglobulin,
whereby a first transfection vector is provided which comprises said provided nucleic acid, which comprises said first as well as said second and/or third nucleic acid, and an additional fourth nucleic acid sequence conferring resistance to a first eukaryotic selection agent, and
whereby a second transfection vector is provided which comprises said provided nucleic acid, which comprises the identical first as well as second and/or third nucleic acid as that/those in said provided nucleic acid contained in the first transfection vector, and an additional fourth nucleic acid sequence conferring resistance to a second eukaryotic selection agent, which is different from the fourth nucleic acid in said first transfection vector, whereby said second eukaryotic selection agent is different from said first eukaryotic selection agent,

c) transfecting said provided CHO cell and selecting said transfected CHO cell with said transfection vectors of step b), wherein said transfecting and selecting comprises the following steps in the following order:
(i) transfecting said CHO cell with said first transfection vector,
(ii) selecting a CHO cell transfected in (i) by selected growth in a cultivation medium containing said first eukaryotic selection agent to which the first transfection vector confers resistance,
(iii) transfecting said CHO cell selected in (ii) with said second transfection vector,
(iv) selecting a CHO cell transfected in (iii) by selected growth in a cultivation medium containing said first eukaryotic selection agent, to which said first transfection vector confers resistance, and containing said second eukaryotic selection agent, to which said second transfection vector confers resistance,

d) cultivating said transfected and selected CHO cell of step c) in a medium containing said first and second eukaryotic selection agent under conditions suitable for the expression of said second and/or third nucleic acid,

e) recovering said secreted heterologous immunoglobulin from the cultivation medium and thereby producing a heterologous immunoglobulin in a CHO cell, which immunoglobulin is secreted to the cultivation medium.

In one embodiment of the method according to the invention said CHO cell is a CHO K1 cell, or a CHO DG44 cell, or a CHO XL99 cell, or a CHO DXB11 cell, or a CHO DP12 cell. In another embodiment the promoter employed for the transcription of said second and third nucleic acids is different from the promoter employed for the transcription of said fourth nucleic acid. A further embodiment is that the promoter employed for the transcription of said second and third nucleic acids is the same. In one embodiment said promoter employed for the transcription of said second and third nucleic acid is the CMV promoter. In another embodiment said promoter employed for the transcription of said fourth nucleic acid is the SV40 promoter. In one embodiment said heterologous immunoglobulin is an anti-Aβ antibody. Exemplary anti-Aβ antibodies are reported e.g. in WO 2003/070760.

In one embodiment said selecting a transfected CHO cell in step c) (ii) and/or (iv) is by growth in cultivation medium without a selection agent for 10 to 72 hours followed by selected growth in a cultivation medium containing said first eukaryotic selection agent in case of (ii) or said first and second eukaryotic selection agent in case of (iv).

In still a further embodiment the codon usage of said second and third nucleic acid is optimized for the translation in CHO cells. Also an embodiment is that said second and/or third nucleic acid contains an intronic nucleic acid sequence. Another embodiment comprises that said first transfection vector and said second transfection vector differ only in the nucleic acid conferring resistance to said eukaryotic selection agent, i.e. in said fourth nucleic acid, and are otherwise at least 95% identical based on the nucleic acid sequence. In another embodiment said transfection vectors differ each only in the nucleic acid conferring resistance to said first, second, and third eukaryotic selection agent.

In one embodiment said method further comprises:
after step b) a step b1):
b1) providing a nucleic acid comprising
a prokaryotic origin of replication,
a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,
a second nucleic acid sequence encoding the heavy chain of said heterologous immunoglobulin, and/or a third nucleic acid sequence encoding the light chain of said heterologous immunoglobulin,
whereby a third transfection vector is provided which comprises said provided nucleic acid, which comprises the identical frist as well as second and/or third nucleic acid as that/those in said provided nucleic acid contained in the first and second transfection vector, and an additional fourth nucleic acid sequence conferring resistance to a third eukaryotic selection agent, which is different from the fourth nucleic acid in said first and second transfection vector, whereby said third eukaryotic selection agent is different from said first eukaryotic selection agent and is also different from said second eukaryotic selection agent,

and further comprises after step c) (iv) the following steps (v) and (vi)
(v) transfecting said CHO cell selected in (iv) with said third transfection vector,
(vi) selecting a CHO cell transfected in (v) by selected growth in cultivation medium containing said first eukaryotic selection agent to which the first transfection vector confers resistance and said second eukaryotic selection agent to which the second transfection vector confers resistance and said third eukaryotic selection agent to which the third transfection vector confers resistance,

and further said medium for cultivating said transfected CHO cell in step d) comprises said first, second, and third eukaryotic selection agent.

In one embodiment said selecting a CHO cell transfected in step c) (vi) is by growth in cultivation medium without a selection agent for 10 to 72 hours followed by selected growth in a cultivation medium containing said first and second and third eukaryotic selection agent.

In another embodiment the method according to the invention comprises a further step

f) purifying said recombinantly produced and recovered heterologous immunoglobulin of step e) with one or more chromatographic steps.

One embodiment is that said step c) and said step d) are performed in the same medium. Still another embodiment is that said medium is a serum-free medium, or a serum-free medium supplemented with defined animal-derived components, or an animal-derived component free medium, or a protein-free medium, or a chemically defined medium, or a defined protein-free medium. In a further embodiment in said step d) is said cultivating in the presence of said eukaryotic selection agents in a volume of less than 500 liter and said cultivating is in the absence of said eukaryotic selection agents in a volume of 500 liter or more, whereby said recovering said secreted heterologous immunoglobulin is from the cultivation medium without said eukaryotic selection agents. In a further embodiment said cultivating in said step d) is comprising sequential cultivations each with increasing cultivation volume up to a preset final cultivation volume, whereby the cultivations are performed in the presence of said eukaryotic selection agents up to a cultivation volume of 1% (v/v) of the cultivation volume of the final cultivation and in the absence of said eukaryotic selection agents in a cultivation volume of more than 1% (v/v) of the cultivation volume of the final cultivation.

The productivity of said CHO cells is in one embodiment over 40 generations not less than 70% and not more than 130% of the productivity after 10 generations of cultivation as split-batch cultivation. In another embodiment is the productivity of said CHO cells over 60 generations not less than 50% and not more than 150% of the productivity after 10 generations of cultivation as split-batch cultivation. In still a further embodiment is the productivity of said CHO cell at least 1.5 g/l of said heterologous immunoglobulin within 21 days as fed-batch cultivation.

A second aspect of the current invention is a CHO cell obtainable with the following method:

a) providing a CHO cell, which is
   adapted to growth in suspension culture,
   adapted to growth in serum-free medium,
   mycoplasma free, and
   optional virus free,

b) providing a nucleic acid comprising
   a prokaryotic origin of replication,
   a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,
   a second nucleic acid sequence encoding the heavy chain of a heterologous immuno globulin, and/or a third nucleic acid sequence encoding the light chain of a heterologous immunoglobulin,
   whereby a first transfection vector is provided which comprises said provided nucleic acid, which comprises said frist as well as second and/or third nucleic acid, and an additional fourth nucleic acid sequence conferring resistance to a first eukaryotic selection agent, and
   whereby a second transfection vector is provided which comprises said provided nucleic acid, which comprises the identical frist as well as second and/or third nucleic acid as that/those in said provided nucleic acid contained in the first transfection vector, and an additional fourth nucleic acid sequence conferring resistance to a second eukaryotic selection agent, which is different from the fourth nucleic acid in said first transfection vector, whereby said second eukaryotic selection agent is different from said first eukaryotic selection agent,

c) transfecting said CHO cell, wherein said transfecting comprises the following steps in the following order:
   (i) transfecting said CHO cell with said first transfection vector,
   (ii) selecting a CHO cell transfected in (i) by selected growth in cultivation medium containing a first eukaryotic selection agent to which the first transfection vector confers resistance,
   (iii) transfecting said CHO cell selected in (ii) with said second transfection vector,
   (iv) selecting a CHO cell transfected in (iii) by selected growth in cultivation medium containing said first eukaryotic selection agent to which the first transfection vector confers resistance and said second eukaryotic selection agent to which the second transfection vector confers resistance.

DETAILED DESCRIPTION OF THE INVENTION

Methods and techniques known to a person skilled in the art, which are useful for carrying out the current invention, are described e.g. in Ausubel, F. M., ed., Current Protocols in Molecular Biology, Volumes I to III (1997), Wiley and Sons; Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

General chromatographic methods and their use are known to a person skilled in the art. See for example, Chromatography, 5th edition, Part A: Fundamentals and Techniques, Heftmann, E. (ed), Elsevier Science Publishing Company, New York, (1992); Advanced Chromatographic and Electromigration Methods in Biosciences, Deyl, Z. (ed.), Elsevier Science By, Amsterdam, The Netherlands, (1998); Chromatography Today, Poole, C. F., and Poole, S. K., Elsevier Science Publishing Company, New York, (1991); Scopes, Protein Purification: Principles and Practice (1982); Sambrook, J., et al. (ed), Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; or Current Protocols in Molecular Biology, Ausubel, F. M., et al. (eds), John Wiley & Sons, Inc., New York.

For the purification of recombinantly produced heterologous immunoglobulins often a combination of different column chromatography steps is employed. Generally a Protein A affinity chromatography is followed by one or two additional separation steps. The final purification step is a so called "polishing step" for the removal of trace impurities and contaminants like aggregated immunoglobulins, residual HCP (host cell protein), DNA (host cell nucleic acid), viruses, or endotoxins. For this polishing step often an anion exchange material in a flow-through mode is used.

Different methods are well established and widespread used for protein recovery and purification, such as affinity chromatography with microbial proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and electrophoretical methods (such as gel electrophoresis, capillary electrophoresis) (Vijayalakshmi, M. A., Appl. Biochem. Biotech. 75 (1998) 93-102).

The term "amino acid" as used within this application denotes the group of carboxy α-amino acids, which directly

or in form of a precursor can be encoded by a nucleic acid. The individual amino acids are encoded by nucleic acids consisting of three nucleotides, so called codons or base-triplets. Each amino acid is encoded by at least one codon. The encoding of the same amino acid by different codons is known as "degeneration of the genetic code". The term "amino acid" as used within this application denotes the naturally occurring carboxy α-amino acids and is comprising alanine (three letter code: ala, one letter code: A), arginine (arg, R), asparagine (asn, N), aspartic acid (asp, D), cysteine (cys, C), glutamine (gln, Q), glutamic acid (glu, E), glycine (gly, G), histidine (his, H), isoleucine (ile, I), leucine (leu, lysine (lys, K), methionine (met, M), phenylalanine (phe, F), proline (pro, P), serine (ser, S), threonine (thr, T), tryptophan (trp, W), tyrosine (tyr, Y), and valine (val, V).

A "nucleic acid" or a "nucleic acid sequence", which terms are used interchangeably within this application, refers to a polymeric molecule consisting of individual nucleotides (also called bases) a, c, g, and t (or u in RNA), for example to DNA, RNA, or modifications thereof. This polynucleotide molecule can be a naturally occurring polynucleotide molecule or a synthetic polynucleotide molecule or a combination of one or more naturally occurring polynucleotide molecules with one or more synthetic polynucleotide molecules. Also encompassed by this definition are naturally occurring polynucleotide molecules in which one or more nucleotides are changed (e.g. by mutagenesis), deleted, or added. A nucleic acid can either be isolated, or integrated in another nucleic acid, e.g. in an expression cassette, a plasmid, or the chromosome of a host cell. A nucleic acid is characterized by its nucleic acid sequence consisting of individual nucleotides.

To a person skilled in the art procedures and methods are well known to convert an amino acid sequence, e.g. of a polypeptide, into a corresponding nucleic acid sequence encoding this amino acid sequence. Therefore, a nucleic acid is characterized by its nucleic acid sequence consisting of individual nucleotides and likewise by the amino acid sequence of a polypeptide encoded thereby.

A "polypeptide" is a polymer consisting of amino acids joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 20 amino acid residues may be referred to as "peptides", whereas molecules consisting of two or more polypeptides or comprising one polypeptide of more than 100 amino acid residues may be referred to as "proteins". A polypeptide may also comprise non-amino acid components, such as carbohydrate groups, metal ions, or carboxylic acid esters. The non-amino acid components may be added by the cell, in which the polypeptide is expressed, and may vary with the type of cell. Polypeptides are defined herein in terms of their amino acid backbone structure or the nucleic acid encoding the same. Additions such as carbohydrate groups are generally not specified, but may be present nonetheless.

The term "immunoglobulin" encompasses the various forms of immunoglobulin structures including complete immunoglobulins and immunoglobulin conjugates. The immunoglobulin employed in the current invention is preferably a human antibody, or a humanized antibody, or a chimeric antibody, or a T cell antigen depleted antibody (see e.g. WO 98/33523, WO 98/52976, and WO 00/34317). Genetic engineering of antibodies is e.g. described in Morrison, S. L., et al., Proc. Natl. Acad Sci. USA 81 (1984) 6851-6855; U.S. Pat. No. 5,202,238 and U.S. Pat. No. 5,204,244; Riechmann, L., et al., Nature 332 (1988) 323-327; Neuberger, M. S., et al., Nature 314 (1985) 268-270; Lonberg, N., Nat. Biotechnol. 23 (2005) 1117-1125. Immunoglobulins may exist in a variety of formats, including, for example, Fv, Fab, and $F(ab)_2$ as well as single chains (scFv) or diabodies (e.g. Huston, J. S., et al., Proc. Natl. Acad. Sci. USA 85 (1988) 5879-5883; Bird, R. E., et al., Science 242 (1988) 423-426; in general, Hood et al., Immunology, Benjamin N.Y., 2nd edition (1984); and Hunkapiller, T. and Hood, L., Nature 323 (1986) 15-16).

The term "complete immunoglobulin" denotes an immuno globulin which comprises two so called light chains and two so called heavy chains. Each of the heavy and light chains of a complete immunoglobulin contains a variable domain (variable region) (generally the amino terminal portion of the polypeptide chain) comprising binding regions that are able to interact with an antigen. Each of the heavy and light chains of a complete immunoglobulin comprises a constant region (generally the carboxyl terminal portion). The constant region of the heavy chain mediates the binding of the antibody i) to cells bearing a Fc gamma receptor (FcγR), such as phagocytic cells, or ii) to cells bearing the neonatal Fc receptor (FcRn) also known as Brambell receptor. It also mediates the binding to some factors including factors of the classical complement system such as component (C1q). The variable domain of an immunoglobulin's light or heavy chain in turn comprises different segments, i.e. four framework regions (FR) and three hypervariable regions (CDR).

The term "immunoglobulin conjugate" denotes a polypeptide comprising at least one domain of an immunoglobulin heavy or light chain conjugated via a peptide bond to a further polypeptide. The further polypeptide is a non-immunoglobulin peptide, such as a hormone, or growth receptor, or antifusogenic peptide, or complement factor, or the like. Exemplary immunoglobulin conjugates are reported in WO 2007/045463.

The term "heterologous immunoglobulin" denotes an immunoglobulin which is not naturally produced by a mammalian cell or the host cell. The immunoglobulin produced according to the method of the invention is produced by recombinant means. Such methods are widely known in the state of the art and comprise protein expression in eukaryotic cells with subsequent recovery and isolation of the heterologous immunoglobulin, and usually purification to a pharmaceutically acceptable purity. For the production, i.e. expression, of an immunoglobulin a nucleic acid encoding the light chain and a nucleic acid encoding the heavy chain are inserted each into an expression cassette by standard methods. Nucleic acids encoding immunoglobulin light and heavy chains are readily isolated and sequenced using conventional procedures. Hybridoma cells can serve as a source of such nucleic acids. The expression cassettes may be inserted into an expression plasmid(s), which is (are) then transfected into host cells, which do not otherwise produce immunoglobulins. Expression is performed in appropriate prokaryotic or eukaryotic host cells and the immunoglobulin is recovered from the cells after lysis or from the culture supernatant.

An "isolated polypeptide" is a polypeptide that is essentially free from contaminating cellular components, such as carbohydrate, lipid, or other proteinaceous impurities associated with the polypeptide in nature. Typically, a preparation of isolated polypeptide contains the polypeptide in a highly purified form, i.e. at least about 80% pure, at least about 90% pure, at least about 95% pure, greater than 95% pure, or greater than 99% pure. One way to show that a particular protein preparation contains an isolated polypeptide is by the appearance of a single band following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of

the protein preparation and Coomassie Brilliant Blue staining of the gel. However, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

"Heterologous DNA" or "heterologous polypeptide" refers to a DNA molecule or a polypeptide, or a population of DNA molecules or a population of polypeptides, that do not exist naturally within a given host cell. DNA molecules heterologous to a particular host cell may contain DNA derived from the host cell species (i.e. endogenous DNA) so long as that host DNA is combined with non-host DNA (i.e. exogenous DNA). For example, a DNA molecule containing a non-host DNA segment encoding a polypeptide operably linked to a host DNA segment comprising a promoter is considered to be a heterologous DNA molecule. Conversely, a heterologous DNA molecule can comprise an endogenous structural gene operably linked with an exogenous promoter.

A peptide or polypeptide encoded by a non-host DNA molecule is a "heterologous" peptide or polypeptide.

The term "cell" or "host cell" refers to a cell into which a nucleic acid, e.g. encoding a heterologous polypeptide, can be or is transfected. The term "cell" includes both prokaryotic cells, which are used for propagation of plasmids, and eukaryotic cells, which are used for the expression of a nucleic acid and production of the encoded polypeptide. In one embodiment, the eukaryotic cells are mammalian cells. In another embodiment the mammalian cell is a CHO cell, preferably a CHO K1 cell (ATCC CCL-61 or DSM ACC 110), or a CHO DG44 cell (also known as CHO-DHFR[−], DSM ACC 126), or a CHO XL99 cell, a CHO-T cell (see e.g. Morgan, D., et al., Biochemistry 26 (1987) 2959-2963), or a CHO-S cell, or a Super-CHO cell (Pak, S. C. O., et al. Cytotechnology. 22 (1996) 139-146). If these cells are not adapted to growth in serum-free medium or in suspension an adaptation prior to the use in the current method is to be performed. As used herein, the expression "cell" includes the subject cell and its progeny. Thus, the words "transformant" and "transformed cell" include the primary subject cell and cultures derived there from without regard for the number of transfers or subcultivations. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

The term "expression" as used herein refers to transcription and/or translation processes occurring within a cell. The level of transcription of a nucleic acid sequence of interest in a cell can be determined on the basis of the amount of corresponding mRNA that is present in the cell. For example, mRNA transcribed from a sequence of interest can be quantitated by RT-PCR or by Northern hybridization (see Sambrook, et al., 1989, supra). Polypeptides encoded by a nucleic acid of interest can be quantitated by various methods, e.g. by ELISA, by assaying for the biological activity of the polypeptide, or by employing assays that are independent of such activity, such as Western blotting or radioimmunoassay, using immunoglobulins that recognize and bind to the polypeptide (see Sambrook, et al., 1989, supra).

An "expression cassette" refers to a construct that contains the necessary regulatory elements, such as promoter and polyadenylation site, for expression of at least the contained nucleic acid in a cell.

A "transfection vector" is a nucleic acid (also denoted as nucleic acid molecule) providing all required elements for the expression of the in the transfection vector comprised coding nucleic acids/structural gene(s) in a host cell. A transfection vector comprises a prokaryotic plasmid propagation unit, e.g. for *E. coli*, in turn comprising a prokaryotic origin of replication, and a nucleic acid conferring resistance to a prokaryotic selection agent, further comprises the transfection vector one or more nucleic acid(s) conferring resistance to an eukaryotic selection agent, and one or more nucleic acid encoding a polypeptide of interest. Preferably are the nucleic acids conferring resistance to a selection agent and the nucleic acid(s) encoding a polypeptide of interest placed each within an expression cassette, whereby each expression cassette comprises a promoter, a coding nucleic acid, and a transcription terminator including a polyadenylation signal. Gene expression is usually placed under the control of a promoter, and such a structural gene is said to be "operably linked to" the promoter. Similarly, a regulatory element and a core promoter are operably linked if the regulatory element modulates the activity of the core promoter.

A "promoter" refers to a polynucleotide sequence that controls transcription of a gene/structural gene or nucleic acid sequence to which it is operably linked. A promoter includes signals for RNA polymerase binding and transcription initiation. The promoter(s) used will be functional in the cell type of the host cell in which expression of the selected sequence is contemplated. A large number of promoters including constitutive, inducible and repressible promoters from a variety of different sources, are well known in the art (and identified in databases such as GenBank) and are available as or within cloned polynucleotides (from, e.g., depositories such as ATCC as well as other commercial or individual sources). A "promoter" comprises a nucleotide sequence that directs the transcription of an operably linked structural gene. Typically, a promoter is located in the 5' non-coding or untranslated region of a gene, proximal to the transcriptional start site of a structural gene. Sequence elements within promoters that function in the initiation of transcription are often characterized by consensus nucleotide sequences. These promoter elements include RNA polymerase binding sites, TATA sequences, CAAT sequences, differentiation-specific elements (DSEs; McGehee, R. E., et al., Mol. Endocrinol. 7 (1993) 551-560), cyclic AMP response elements (CREs), serum response elements (SREs; Treisman, R., Seminars in Cancer Biol. 1 (1990) 47-58), glucocorticoid response elements (GREs), and binding sites for other transcription factors, such as CRE/ATF (O'Reilly, M. A., et al., J. Biol. Chem. 267 (1992) 19938-19943), AP2 (Ye, J., et al., J. Biol. Chem. 269 (1994) 25728-25734), SP1, cAMP response element binding protein (CREB; Loeken, M. R., Gene Expr. 3 (1993) 253-264) and octamer factors (see, in general, Watson et al., eds., Molecular Biology of the Gene, 4th ed. (The Benjamin/Cummings Publishing Company, Inc. 1987), and Lemaigre, F. P. and Rousseau, G. G., Biochem. J. 303 (1994) 1-14). Among the eukaryotic promoters that have been identified as strong promoters for high-level expression are the SV40 early promoter, adenovirus major late promoter, mouse metallothionein-I promoter, Rous sarcoma virus long terminal repeat, Chinese hamster elongation factor 1 alpha (CHEF-1, see e.g. U.S. Pat. No. 5,888,809), human EF-1 alpha, ubiquitin, and human cytomegalovirus immediate early promoter (CMV IE).

The "promoter" can be constitutive or inducible. An enhancer (i.e., a cis-acting DNA element that acts on a promoter to increase transcription) may be necessary to function in conjunction with the promoter to increase the level of expression obtained with a promoter alone, and may be included as a transcriptional regulatory element. Often,

the polynucleotide segment containing the promoter will include enhancer sequences as well (e.g., CMV or SV40).

An "enhancer", as used herein, refers to a polynucleotide sequence that enhances transcription of a gene or coding sequence to which it is operably linked. Unlike promoters, enhancers are relatively orientation and position independent and have been found 5' or 3' (Lusky, M., et al., Mol. Cell Bio., 3 (1983) 1108-1122) to the transcription unit, within an intron (Banerji, J., et al., Cell, 33 (1983) 729-740) as well as within the coding sequence itself (Osborne, T. F., et al., Mol. Cell. Bio., 4 (1984) 1293-1305). Therefore, enhancers may be placed upstream or downstream from the transcription initiation site or at considerable distances from the promoter, although in practice enhancers may overlap physically and functionally with promoters. A large number of enhancers, from a variety of different sources are well known in the art (and identified in databases such as GenBank) and are available as or within cloned polynucleotide sequences (from, e.g., depositories such as the ATCC as well as other commercial or individual sources). A number of polynucleotides comprising promoter sequences (such as the commonly-used CMV promoter) also comprise enhancer sequences. For example, all of the strong promoters listed above may also contain strong enhancers (see e.g. Bendig, M., Genetic Engineering 7 (Academic Press, 1988) 91-127).

A "nucleic acid conferring resistance to a selection agent" is a nucleic acid that allows cells carrying it to be specifically selected for or against, in the presence of a selection agent. Such a nucleic acid is also denoted as selection marker. Typically, a selection marker will confer resistance to a selection agent (drug) or compensate for a metabolic or catabolic defect in the host cell. A selection marker can be positive, negative, or bifunctional. A useful positive selection marker is an antibiotic resistance gene. This selection marker allows cells transformed therewith to be positively selected for in the presence of the corresponding selection agent, i.e. under selected growth in the presence e.g. of the corresponding antibiotic. A non-transformed cell is not capable to grow or survive under the selective growth conditions, i.e. in the presence of the selection agent, in culture. Positive selection markers allow selection for cells carrying the marker, whereas negative selection markers allow cells carrying the marker to be selectively eliminated. Eukaryotic selection markers include, e.g., the genes for aminoglycoside phosphotransferase (APH) (conferring resistance to the selection agents such as e.g. hygromycin (hyg), neomycin (neomycin phosphotransferase II, neo), and G418), dihydrofolate reductase (DHFR) (conferring resistance to the selection agent methotrexate), thymidine kinase (tk), glutamine synthetase (GS), asparagine synthetase, tryptophan synthetase (conferring resistance to the selection agent indole), histidinol dehydrogenase (conferring resistance to the selection agent histidinol D), cytidine deaminase, adenosine deaminase and nucleic acids conferring resistance to puromycin, bleomycin, phleomycin, chloramphenicol, Zeocin, and mycophenolic acid. Further selection marker nucleic acids are reported e.g. in WO 92/08796 and WO 94/28143. Prokaryotic selection markers include, e.g. the beta-lactamase gene (conferring resistance to the selection agent ampicillin).

Expression of a gene is performed either as transient or as permanent expression. The polypeptide(s) of interest are in general secreted polypeptides and therefore contain an N-terminal extension (also known as the signal sequence) which is necessary for the transport/secretion of the polypeptide through the cell wall into the extracellular medium. In general, the signal sequence can be derived from any gene encoding a secreted polypeptide. If a heterologous signal sequence is used, it preferably is one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For secretion in yeast for example the native signal sequence of a heterologous gene to be expressed may be substituted by a homologous yeast signal sequence derived from a secreted gene, such as the yeast invertase signal sequence, alpha-factor leader (including *Saccharomyces, Kluyveromyces, Pichia*, and *Hansenula* α-factor leaders, the second described in U.S. Pat. No. 5,010,182), acid phosphatase signal sequence, or the *C. albicans* glucoamylase signal sequence (EP 0 362 179). In mammalian cell expression the native signal sequence of the protein of interest is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from secreted polypeptides of the same or related species, e.g. for immunoglobulins from human or murine origin, as well as viral secretory signal sequences, for example, the herpes simplex glycoprotein D signal sequence. The DNA fragment encoding for such a presegment is ligated in frame, i.e. operably linked, to the DNA fragment encoding a polypeptide of interest.

The first aspect of the current invention is a method for the recombinant production of a secreted heterologous immunoglobulin in a CHO cell which comprises:

a) providing a CHO cell, which is adapted to growth in suspension culture, adapted to growth in serum-free medium, and mycoplasma free;

b) providing a transfection vector, which comprises the following elements:
  - a prokaryotic origin of replication,
  - a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,
  - a second nucleic acid sequence encoding the heavy chain of said heterologous immunoglobulin and a third nucleic acid sequence encoding the light chain of said heterologous immunoglobulin,
  - a fourth nucleic acid sequence conferring resistance to a eukaryotic selection agent,
  - whereby each of said first to fourth nucleic acid sequence is contained in an expression cassette,

c) transfecting and selecting said CHO cell, wherein said transfecting and selecting comprises the following steps in the following order:
  - (i) transfecting said CHO cell with a transfection vector comprising said first to third nucleic acid and a fourth nucleic acid sequence conferring resistance to a first eukaryotic selection agent,
  - (ii) selecting a CHO cell transfected in (i) by selected growth in cultivation medium containing said first eukaryotic selection agent,
  - (iii) transfecting said CHO cell selected in (ii) with a transfection vector comprising said first to third nucleic acid and a fourth nucleic acid sequence different from that in the transfection vector used in (i) conferring resistance to a second eukaryotic selection agent different to said first eukaryotic selection agent,
  - (iv) selecting a CHO cell transfected in (iii) by selected growth in cultivation medium containing said first and said second eukaryotic selection agent,

d) cultivating said transfected and selected CHO cell of step c) in a cultivation medium containing said first and second eukaryotic selection agent, under conditions suitable for the expression of said second, and third nucleic acid,

e) recovering said secreted heterologous immunoglobulin from the cultivation medium and thereby recombinantly producing a heterologous immunoglobulin.

The method according to the invention is suited for the production of a secreted heterologous immunoglobulin in large scale, i.e. industrially. The cultivation of a cell for the production of a desired polypeptide in large scale generally consists of a sequence of individual cultivations, wherein all cultivations except the final, i.e. the large scale, cultivation, i.e. the last one in the sequence, are performed until a certain cell density is reached in the culture vessel. If the predetermined cell density is reached the entire cultivation or a fraction thereof is used to inoculate the next cultivation vessel, which has a larger volume, up to 1000 times the volume of the preceding cultivation. All cultivations which serve as a basis for at least one further cultivation in a larger volume are denoted as seed train fermentations. Only in the large scale cultivation, i.e. in the cultivation which is not intended to serve as the basis for a further cultivation in a larger volume, which is also denoted as main fermentation, is the endpoint of the cultivation determined depending on the concentration of the produced secreted heterologous immunoglobulin in the cultivation medium. The term "large scale" as used within this application denotes the final cultivation of an industrial production process. Preferably a large scale cultivation is performed at a volume of at least 100 l, more preferably of at least 500 l, most preferably of at least 1000 l up to a volume of 20,000 l. In one embodiment the final, i.e. large scale, cultivation medium does not contain a eukaryotic selection agent.

In one embodiment the cultivation of said transfected CHO cell is performed in the presence of said eukaryotic selection agent in a volume of less than 500 liter and the cultivation of said transfected CHO cell is performed in the absence of said eukaryotic selection agents in a volume of 500 liter or more and that said recovering said secreted heterologous immunoglobulin is from the cultivation medium without said eukaryotic selection agents. In a further embodiment the cultivation is comprising sequential cultivations with increasing cultivation volume up to a final cultivation volume, whereby the cultivations are performed in the presence of said eukaryotic selection agents up to a cultivation volume of 1% (v/v) of the cultivation volume of the final or main cultivation and in the absence of all of said eukaryotic selection agents in a cultivation volume of more than 1% (v/v) of the cultivation volume of the final cultivation. In a further embodiment said cultivation is comprising sequential seed train cultivations with increasing cultivation volume, whereby each of the seed train cultivations is performed in the presence of said eukaryotic selection agents and the main fermentation is performed in the absence of all of said eukaryotic selection agents. In one embodiment the cultivation of said transfected CHO cell is performed in the presence of said eukaryotic selection agent in the seed train fermentations and the cultivation of said transfected CHO cell is performed in the absence of said eukaryotic selection agents in the main fermentation and that said recovering said secreted heterologous immunoglobulin is from the main cultivation medium not containing said eukaryotic selection agents. In these embodiments the eukaryotic selection agents are added during the growth phase and omitted during the production phase of said CHO cell. The term "production phase" denotes the cultivation of a CHO cell in a large volume, i.e. the main fermentation, after which the produced heterologous immunoglobulin is recovered.

In another embodiment of the method according to the invention the productivity of said CHO cell is over 40 generations not less than 70% and not more than 130% of the productivity after 10 generations of cultivation as split-batch cultivation. In an embodiment the productivity of said CHO cells is over 60 generations not less than 50% and not more than 150% of the productivity after 10 generations of cultivation as split-batch cultivation. The productivity of said CHO cell is at least 1.5 g/l of said heterologous immunoglobulin within 21 days as fed-batch cultivation in another embodiment. In one embodiment the specific productivity of the CHO cell obtained with the method according to the invention is more than 1 μg/10^6 cells/d, more than 5 μg/10^6 cells/d, or more than 10 μg/10^6 cells/d. In one embodiment the secreted heterologous immunoglobulin is a completely processed secreted heterologous immunoglobulin. The term "completely processed secreted heterologous immunoglobulin" denotes an immunoglobulin i) which is secreted to the cultivation medium and whose signal sequences has been cleaved, ii) which comprises an antigen binding region, iii) which has secondary modifications, such as attached saccharides or polysaccharides, and/or correctly formed disulfide bonds.

In one embodiment of the invention the heterologous immunoglobulin is an anti-Aβ antibody. In another embodiment the heavy chain variable domain of said anti-Aβ antibody comprises a CDR3 with an amino acid sequence selected from SEQ ID NO: 1, 2, or 3. In a further embodiment the light chain variable domain of said anti-Aβ antibody comprises a CDR3 with an amino acid sequence selected from SEQ ID NO: 4, 5, or 6. In a further embodiment said anti-Aβ antibody comprises a heavy chain variable domain with an amino acid sequence selected from SEQ ID NO: 7, 8, or 9. In still a further embodiment said anti-Aβ antibody comprises a light chain variable domain with an amino acid sequence selected from SEQ ID NO: 10, 11, or 12.

In one embodiment of the invention the heterologous immunoglobulin is an anti-P-Selectin antibody. In a further embodiment said anti-P-Selectin antibody comprises a heavy chain variable domain with an amino acid sequence selected from SEQ ID NO: 13, 14, or 15. In still a further embodiment said anti-P-Selectin antibody comprises a light chain variable domain with an amino acid sequence selected from SEQ ID NO: 16, 17, or 18.

In one embodiment of the invention the heterologous immunoglobulin is an anti-IL-13Rα antibody. In a further embodiment said anti-IL-13Rα antibody comprises a heavy chain variable domain with an amino acid sequence selected from SEQ ID NO: 19, 20, 21, 22, or 23. In still a further embodiment said anti-IL-13Rα antibody comprises a light chain variable domain with an amino acid sequence selected from SEQ ID NO: 24, 25, 26, 27, or 28.

In one embodiment of the invention the heterologous immunoglobulin is an anti-CD4 antibody-conjugate. In another embodiment the heavy chain variable domain of said anti-CD4 antibody in said conjugate comprises a CDR3 with an amino acid sequence selected from SEQ ID NO: 29, 30, or 31. In a further embodiment the light chain variable domain of said anti-CD4 antibody in said conjugate comprises a CDR3 with an amino acid sequence selected from SEQ ID NO: 32, 33, or 34. In a further embodiment said anti-CD4 antibody in said conjugate comprises a heavy chain variable domain with an amino acid sequence selected from SEQ ID NO: 35, 36, or 37. In still a further embodiment said anti-CD4 antibody in said conjugate comprises a light chain variable domain with an amino acid sequence selected from SEQ ID NO: 38, 39, or 40.

A mammalian cell usable for the large scale production of therapeutics, i.e. polypeptides intended for the use in humans, has to fulfill distinct criteria. Amongst others are

these that it has to be cultivatable in serum-free, preferably in non-defined mammal-derived components free medium, or in a serum-free medium supplemented with defined mammal-derived components. Serum is a mixture of multitude of compounds. Normally bovine serum has been used for the cultivation of mammalian cells. With the arising problem of transmissible diseases from one species to another the use of serum and other non-defined mammal-derived compounds has to be avoided. The term "non-defined mammal-derived compound" as used within this application denotes compounds which are derived from a mammal, especially preferred from a cow, a pig, a sheep, or a lamb, and whose composition can be specified to less than 80%, preferably to less than 90% (w/w). A "defined mammal-derived compound" is a compound that is obtained from a mammal, especially preferred from a cow, a pig, a sheep, or a lamb, and whose composition can be specified to more than 95% (w/w), preferably to more than 98% (w/w), most preferably to more then 99% (w/w). An example of a defined mammal-derived compound is cholesterol from ovine wool, and galactose from bovine milk. In one embodiment the medium can be supplemented with defined or non-defined not mammal-derived compounds. An example of such a not mammal-derived compound is cod-liver oil.

Therefore in one embodiment of the current invention the medium used in the cultivation is a serum-free medium, or a serum-free medium supplemented with defined mammal-derived components, or an mammal-derived component free medium, or a protein-free medium, a protein-free medium supplemented with defined mammal-derived components, or a chemically defined medium, or a mammal-derived component free medium, or a defined protein-free medium. Examples of an mammal-derived component free medium are the CD CHO medium available from Invitrogen Corp., or the ProCHO4 available from Gibco. An example of a protein free medium is HyQ SFM4CHO available from Hyclone.

In another embodiment of the method according to the invention is the method beginning with the first transfection and ending with the recovery of the secreted heterologous immunoglobulin performed in the same medium. The term "in the same medium" denotes within the current application that beginning with the first transfection and ending with the recovery of the secreted heterologous immunoglobulin from the cultivation medium the same medium is used. This does not denote that the same additives have to be added to the medium in all steps, i.e. the medium may be supplemented with different additive in different steps of the method. Additives are compounds that are added to a medium in total to less than 20% (w/w), in one embodiment to less than 15% (w/w), in another embodiment to less than 10% (w/w). In one embodiment the medium used in the method according to the invention is the same medium in all steps and is a medium suitable for the large scale production of the secreted heterologous immunoglobulin.

It has surprisingly been found that with the method according to the invention a multiple transfected CHO cell can be obtained that has similar growth characteristics and an improved productivity compared to a one-time transfected CHO cell. The term "similar growth characteristics" denotes that the multiple transfected CHO cell grows to at least 50% of the cell density within the same time as the one-time transfected CHO cell. In another embodiment said multiple transfected CHO cell grows to at least 90% of the cell density as the one-time transfected cell. In still a further embodiment is the doubling time of the multiple transfected cell at most 150% of that of the one-time transfected cell. In one embodiment said multiple transfected CHO cell is a CHO cell transfected two or three times. In another embodiment the multiple transfected cell has an improved volumetric yield in a cultivation medium. The overall productivity of a large scale fermentation process is best determined by the volumetric yield, i.e. the amount of polypeptide per unit volume of the cultivation. This volumetric yield is the product of cell density, specific productivity of each cell and cultivation time. Thus, a cultivation with low cell density but high specific productivity will have the same volumetric yield in the same time as a cultivation with high cell density but low specific productivity in the same cultivation time. Thus, with the multiple transfected CHO cell and the method according to the invention a CHO cell is obtainable with similar growth characteristics but an improved volumetric yield/productivity compared to one-time transfected CHO cells.

The secreted heterologous immunoglobulin can be recovered from the cultivation medium with chromatographic methods known to a person of skill in the art. Therefore in one embodiment the method according to the invention comprises the final step of purifying said heterologous immunoglobulin with one or more chromatographic steps.

A vector suited for use in the method according to the invention comprises a prokaryotic origin of replication, and a first nucleic acid conferring resistance to a prokaryotic selection agent, and/or a second nucleic acid encoding the heavy chain of said heterologous immunoglobulin, and/or a third nucleic acid encoding the light chain of said heterologous immunoglobulin, and a fourth nucleic acid conferring resistance to a eukaryotic selection agent.

The comprised first nucleic acid confers resistance to the addition of a prokaryotic selection agent to the cultivation medium. Exemplary prokaryotic selection agents are e.g. ampicillin, kanamycin, chloramphenicol, tetracycline, or erythromycin. The term "a nucleic acid conferring resistance to a selection agent" and grammatical equivalents thereof denotes within the current application that the polypeptide encoded by said nucleic acid can neutralize said selection agent by modification or degradation or can counteract the effect of said selection agent. Thus, a cell comprising a nucleic acid conferring resistance to a selection agent has the ability to survive and proliferate with the selection agent present in the cultivation medium. Exemplary eukaryotic selection agents are e.g. neomycin, hygromycin, puromycin, methotrexate, Geneticin® (G418), or mycophenolic acid. The selection agent is chosen with the proviso that the prokaryotic and the eukaryotic selection agent is not a metal.

The transfection of the provided CHO cell according to the method according to the invention is performed as sequential steps of transfection and selection. CHO cells suitable in the method according to the invention are e.g. a CHO K1 cell, or a CHO DG44 cell, or a CHO XL99 cell, or a CHO DXB11 cell, or a CHO DP12 cell, or a super-CHO cell. Within the scope of the present invention, transfected cells may be obtained with substantially any kind of transfection method known in the art. For example, the nucleic acid may be introduced into the cells by means of electroporation or microinjection. Alternatively, lipofection reagents such as FuGENE 6 (Roche Diagnostics GmbH, Germany), X-tremeGENE (Roche Diagnostics GmbH, Germany), LipofectAmine (Invitrogen Corp., USA), and nucleotransfection (AMAX Corp.) may be used. Still alternatively, the nucleic acid may be introduced into the cell by appropriate viral vector systems based on retroviruses, lentiviruses, adenoviruses, or adeno-associated viruses (Singer, O., Proc. Natl. Acad. Sci. USA 101 (2004) 5313-5314).

After the transfection positive transfected cells are selected in the presence of selection agents, i.e. by selected growth. It has surprisingly been found that more than one eukaryotic selection agent can be present in the cultivation medium not interfering with growth and heterologous polypeptide expression if the cultivated CHO cell has been transfected with all required corresponding nucleic acids conferring resistance to these eukaryotic selection agents according to the current invention. It has also been found that CHO cells can be cultivated in the concomitant presence of three eukaryotic selection agents without a reduction of the doubling time to more than 150% of the doubling time of the non-transfected or one-time transfected CHO cell. Therefore, the multiple transfected CHO cell comprises nucleic acids, which are in each transfection step of the method according to the invention comprising a different, not previously transfected, nucleic acid as fourth nucleic acid which confers a new resistance not already present in said CHO cell to a different eukaryotic selection agent. Therefore, after the second transfection step a successfully transfected cell is selected for by cultivation in the concomitant presence of two different eukaryotic selection agents. After the third transfection the transfected cell can be cultivated for selection in the concomitant presence of three different eukaryotic selection agents.

Thus, the vector employed in the different transfection steps according to the method according to the invention is at least 95% identical on the nucleic acid level except for the nucleic acid conferring resistance to a eukaryotic selection agent, i.e. the fourth nucleic acid.

For the expression of a secreted heterologous immunoglobulin the vector with which the CHO cell is transfected and which comprises a nucleic acid conferring resistance to a eukaryotic selection agent also comprises a nucleic acid encoding the light chain of said heterologous immunoglobulin and/or a nucleic acid encoding the heavy chain of said heterologous immunoglobulin. If the vector comprises only a nucleic acid encoding either the light chain of said immunoglobulin or the heavy chain of said immunoglobulin said CHO cell is also transfected in each step by another vector comprising a nucleic acid encoding the corresponding other chain of said immunoglobulin.

In one embodiment the first to fourth nucleic acid sequence comprised in the transfection vectors according to the invention (i.e. the first, second, and third transfection vector) is contained in an expression cassette. An “expression cassette” refers to a construct that contains the necessary regulatory elements, such as promoter and polyadenylation site, for expression of at least the contained nucleic acid in a cell, e.g. a promoter, a nucleic acid to be expressed, and a transcription terminator including a polyadenylation signal. The promoter contained in the expression cassette determines the amount of transcription of the operably linked nucleic acid and therewith it determines the amount of the translation of said nucleic acid. A first promoter inducing a larger amount of translation of a nucleic acid compared to a second promoter is termed a “stronger promoter” with respect to said second promoter. It is intended to produce the secreted heterologous immunoglobulin and not the polypeptide conferring resistance to a selection agent. Thus, the capacity of the host cells transcription and translation machinery has to be split up correspondingly. Therefore, in one embodiment the promoter employed for the transcription of said second and third nucleic acids is different from the promoter employed for the transcription of said fourth nucleic acid. In another embodiment is the amount of transcript of said second and third nucleic acid encoding the chains of said heterologous immunoglobulin larger than the amount of transcript of said forth nucleic acid conferring resistance to a selection agent. Thus, the promoter employed for the expression of said second and third nucleic acid is stronger than the promoter employed for the expression of said fourth nucleic acid. In another embodiment is the promoter employed for the transcription of said second and third nucleic acids the same but different from the promoter of said fourth nucleic acid. In one embodiment the promoter for the expression of said second and third nucleic acid is the CMV promoter or a variant thereof and the promoter for the expression of said fourth nucleic acid is the SV40 promoter or a variant thereof.

In a further embodiment of the method according to the invention the codon usage of said second and third nucleic acid is optimized for the expression in CHO cells. This allows a more efficient use of the transfer-RNAs present in the recombinant CHO cell. In another embodiment said second and/or third nucleic acid comprise an intronic nucleic acid sequence, in another embodiment the intronic nucleic acid is a mouse/human hybrid intron. In the genome of eukaryotic cells the genomic DNA sequences contain coding (exonic) and non-coding (intronic) nucleic acid sequences. After transcription of the DNA to the pre-mRNA, the pre-mRNA also contains these intronic and exonic nucleic acid sequences. Prior to translation the non-coding intronic nucleic acid sequences are removed during mRNA processing by splicing them out of the primary mRNA transcript to generate the mature mRNA. The splicing of the primary mRNA is controlled by a splice donor site in combination with a properly spaced apart splice acceptor site. The splice donor site is located at the 5' end and the splice acceptor site is located at the 3' end of an intronic sequence and both are only partly removed during the pre-mRNA splicing.

To produce a secreted polypeptide, the nucleic acid(s) encoding the chains of the heterologous immunoglobulin include a DNA segment that encodes a signal sequence/leader peptide. The signal sequence directs the newly synthesized polypeptide to and through the Endoplasmatic reticulum (ER) membrane where the polypeptide can be routed for secretion. The signal sequence is cleaved off by a signal peptidases during crossing of the ER membrane. As for the function of the signal sequence the recognition by the host cell’s secretion machinery is essential. Therefore, the used signal sequence has to be recognized by the host cell’s proteins and enzymes of the secretion machinery.

In one embodiment the method according to the invention comprises a third transfection step in step c):

(v) transfecting said CHO cell selected in (iv) with said vector comprising a fourth nucleic acid sequence different from that in the transfection vector used in (i) and (iii) conferring resistance to a third eukaryotic selection agent, which is different from said first and said second eukaryotic selection agent,

(vi) selecting a CHO cell transfected in (v) by selected growth in a cultivation medium containing said first and said second and said third eukaryotic selection agent.

In this embodiment the cultivation medium employed for the cultivation of said transfected CHO cell in step d) further comprises a third eukaryotic selection agent.

A second aspect of the current invention is a CHO cell expressing a secreted heterologous immunoglobulin obtainable with the following method:

a) providing a CHO cell, which is
adapted to growth in suspension culture,
adapted to growth in serum-free medium,
mycoplasma free,

b) providing a nucleic acid comprising
a prokaryotic origin of replication,
a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,
a second nucleic acid sequence encoding the heavy chain of said heterologous immunoglobulin, and a third nucleic acid sequence encoding the light chain of said heterologous immunoglobulin,
whereby a first transfection vector is provided which comprises said provided nucleic acid and an additional fourth nucleic acid sequence conferring resistance to a first eukaryotic selection agent,
whereby a second transfection vector is provided which comprises said provided nucleic acid and an additional fourth nucleic acid sequence different from the fourth nucleic acid in said first transfection vector conferring resistance to a second eukaryotic selection agent, whereby said second eukaryotic selection agent is different to said first eukaryotic selection agent,

c) transfecting and selecting said CHO cell, wherein said transfecting and selecting comprises the following steps in the following order:
(i) transfecting said CHO cell with said first transfection vector,
(ii) selecting a CHO cell transfected in (i) by selected growth in a cultivation medium containing a first eukaryotic selection agent to which the first transfection vector confers resistance,
(iii) transfecting said CHO cell selected in (ii) with said second transfection vector,
(iv) selecting a CHO cell transfected in (iii) by selected growth in a cultivation medium containing said first eukaryotic selection agent, to which the first transfection vector confers resistance, and said second eukaryotic selection agent, to which the second transfection vector confers resistance.

The term “virus free” which is used within this application denotes that the CHO cell does not contain any viral nucleic acid which would result if expressed during cultivation in harmful, in down stream processing operations not separatable products for humans.

The following examples, and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

DESCRIPTION OF THE FIGURES

FIG. **1** Annotated plasmid map of plasmid p5128.
FIG. **2** Annotated plasmid map of plasmid p5137.
FIG. **3** Annotated plasmid map of plasmid p5151.
FIG. **4** Annotated plasmid map of plasmid p5057.
FIG. **5** Annotated plasmid map of plasmid p5069.

FIG. **6** (A) Antibody titers of clones obtained after subcloning with limited dilution and of clones obtained with the method according to the invention; X-axis: (1) G24, (2) limited dilution, (3) method according to the invention; Y-axis: immunoglobulin concentration [μg/ml].

(B) Specific production rates of clones obtained after subcloning with limited dilution and of clones obtained with the method according to the invention; X-axis: (1) G24, (2) limited dilution, (3) method according to the invention; Y-axis: specific production rate [pg/d*cell].

FIG. **7** SDS-Page after protein-A HPLC purification of the antibody. For the four samples 35-45, 37-65, 39-4 and 43-16 two bands are visible, the upper being the heavy chain, the lower being the light chain. Sample 25g7 is a control antibody with antibody-related side products (above the heavy chain and between heavy and light chain). Samples: (1) Molecular weight marker, (2) 35-45, (3) 37-65, (4) 39-4, (5) 43-16), (6) 25g7, (7) Reference antibody, (8) Medium 25×.

FIG. **8** Annotated plasmid map of plasmid p6311.
FIG. **9** Annotated plasmid map of plasmid p5321.

EXAMPLES

Materials & Methods

General information regarding the nucleotide sequences of human immuno globulins light and heavy chains is given in: Kabat, E. A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, Md. (1991). Amino acids of antibody chains are numbered according to EU numbering (Edelman, G. M., et al., Proc. Natl. Acad. Sci. USA 63 (1969) 78-85; Kabat, E. A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, Md., (1991)).

Recombinant DNA Techniques:

Standard methods were used to manipulate DNA as described in Sambrook, J., et al., Molecular cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989. The molecular biological reagents were used according to the manufacturer’s instructions.

Gene Synthesis:

Desired gene segments were prepared from oligonucleotides made by chemical synthesis. The 100-600 bp long gene segments, which are flanked by singular restriction endonuclease cleavage sites, were assembled by annealing and ligation of oligonucleotides including PCR amplification and subsequently cloned into the pCR2.1-TOPO-TA cloning vector (Invitrogen Corp., USA) via A-overhangs or pPCR-Script Amp SK(+) cloning vector (Stratagene Corp., USA). The DNA sequence of the subcloned gene fragments were confirmed by DNA sequencing.

Protein Determination:

Protein concentration was determined by determining the optical density (OD) at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence.

Antibody Titer Determination:

Antibody titers were determined either by anti-human Fc ELISA or by Protein A chromatography using the autologous purified antibody as a reference.

SDS-PAGE

LDS sample buffer, fourfold concentrate (4×): 4 g glycerol, 0.682 g TRIS-Base, 0.666 g TRIS-hydrochloride, 0.8 g LDS (lithium dodecyl sulfate), 0.006 g EDTA (ethylene diamin tetra acid), 0.75 ml of a 1% by weight (w/w) solution of Serva Blue G250 in water, 0.75 ml of a 1% by weight (w/w) solution of phenol red, add water to make a total volume of 10 ml.

The culture broth containing the secreted antibody was centrifuged to remove cells and cell debris. An aliquot of the clarified supernatant was admixed with ¼ volumes (v/v) of 4×LDS sample buffer and 1/10 volume (v/v) of 0.5 M

1,4-dithiotreitol (DTT). Then the samples were incubated for 10 min. at 70° C. and protein separated by SDS-PAGE. The NuPAGE® Pre-Cast gel system (Invitrogen Corp.) was used according to the manufacturer's instruction. In particular, 10% NuPAGE® Novex® Bis-TRIS Pre-Cast gels (pH 6.4) and a NuPAGE® MOPS running buffer was used.

Western Blot

Transfer buffer: 39 mM glycine, 48 mM TRIS-hydrochloride, 0.04% by weight (w/w) SDS, and 20% by volume methanol (v/v)

After SDS-PAGE the separated antibody chains were transferred electrophoretically to a nitrocellulose filter membrane (pore size: 0.45 μm) according to the "Semidry-Blotting-Method" of Burnette (Burnette, W. N., Anal. Biochem. 112 (1981) 195-203).

Example 1

Expression Vector for Expressing an Anti-Aβ Antibody

An example (preferably monoclonal) antibody for which a cell line for expression can be obtained according to the current invention is an antibody against the amyloid β-A4 peptide (anti-Aβ antibody). Such an antibody and the corresponding nucleic acid sequences are, for example, reported in WO 2003/070760 or US 2005/0169925 or in SEQ ID NO: 1 to 12.

The anti-Aβ antibody expressing Chinese hamster ovary (CHO) cell line was generated by three successive complete transfections and selection campaigns.

A genomic human κ-light chain constant region gene segment (C-kappa, C_L) was added to the light chain variable region of the anti-Aβ antibody, while a human γ1-heavy chain constant region gene segment (C_{H1}-Hinge-C_{H2}-C_{H3}) was added to the heavy chain variable region of the anti-Aβ antibody. The complete κ-light and γ1-heavy chain antibody genes were then joined with a human cytomegalovirus (HCMV) promoter at the 5'-end and a human immunoglobulin polyadenylation signal sequence at the 3'-end.

a) Heavy Chain Expression Cassette

The transcription unit of the anti-Aβ antibody heavy chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus,
- a 5'-untranslated region derived from a human antibody germline gene,
- the anti-Aβ antibody heavy chain variable domain including a signal sequence derived from a human antibody germline gene,
- a human/mouse heavy chain hybrid intron 2 including the mouse Ig heavy chain enhancer element (see e.g. (Neuberger, M. S., EMBO J. 2 (1983) 1373-1378),
- the genomic human γ1-heavy chain gene constant region,
- the human immunoglobulin γ1-heavy chain polyadenylation ("poly A") signal sequence,
- the unique restriction sites AscI and SgrAI at the 5'- and 3'-end, respectively.

b) Light Chain Expression Cassette

The transcription unit of the anti-Aβ antibody light chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (HCMV),
- a 5'-untranslated region derived from a human antibody germline gene,

- the anti-Aβ antibody light chain variable region including a signal sequence derived from a human antibody germline gene,
- a human/mouse κ-light gene hybrid intron 2 including the mouse Ig χ-light chain enhancer element (Picard and Schaffner, A lymphocyte-specific enhancer in the mouse immunoglobulin kappa gene. Nature 307 (1984) 80-82),
- the human κ-light gene constant region (C-kappa),
- the human immunoglobulin κ-polyadenylation ("poly A") signal sequence,
- the unique restriction sites Sse8387 and FseI at the 5'- and 3'-end, respectively.

c) Expression Plasmids 5128, 5137, and 5151

For expression and production of the anti-Aβ antibody the light and heavy chain expression cassettes were placed on a single expression vector (heavy chain upstream of light chain in clockwise orientation). Three identical expression vectors were generated differing only in the selectable marker gene included, in particular, in the gene conferring resistance to the selection agent neomycin, hygromycin, or puromycin. The vectors also include a mouse DHFR gene which was not used for selection or amplification.

The expression vectors contain beside the light and heavy chain expression cassette the following elements:

- a selectable marker (either a neomycin, hygromycin or puromycin resistance gene),
- an origin of replication allowing for the replication of the plasmid in *E. coli,*
- a beta-lactamase gene which confers ampicillin resistance in *E. coli,*
- a mouse derived DHFR gene.

The plasmid map of the expression vector 5128 containing a hygromycin selectable marker gene is shown in FIG. **1**. The plasmid map of the expression vector 5137 containing a neomycin selectable marker gene is shown in FIG. **2**. The plasmid map of the expression vector 5151 containing a puromycin selectable marker gene is shown in FIG. **3**.

Example 2

Transfection and Selection of a CHO Cell Expressing an Anti-Aβ Antibody

Parent CHO-K1 cells, pre-adapted to growth in serum-free suspension culture in synthetic animal component free ProCHO4 medium (Cambrex Corp.) containing 8 mM glutamine and 1×HT supplement (Gibco/Invitrogen) were used as host cell line. This supplemented ProCHO4 medium is designated in the following as ProCHO4-complete medium. The adherent growing CHO-K1 parent cell line was received from ATTC as ATCC CCL-61.

The preadapted parent host cells were propagated in suspension in synthetic, animal component-free ProCHO4-complete medium under standard humidified conditions (95%, 37° C., and 5% CO_2). On regular intervals depending on the cell density the cells were splitted into fresh medium. The cells were harvested by centrifugation in the exponential growth phase, washed once in sterile Phosphate Buffered Saline (PBS) and resuspended in sterile PBS.

Prior to transfection the anti-Aβ antibody expressing plasmids were linearized within the β-lactamase gene (*E. coli* ampicillin resistance marker gene) using the restriction endonuclease enzyme PvuI or AviII. The cleaved DNA was precipitated with ethanol, dried under vacuum, and dissolved in sterile PBS.

In general, for transfection, the (parent or already transfected) CHO cells were electroporated with 20-50 μg linearized plasmid DNA per approximately 10^7 cells in PBS at room temperature. The electroporations were performed with a Gene Pulser XCell electroporation device (Bio-Rad Laboratories) in a 2 mm gap cuvette, using a square wave protocol with a single 180 V pulse. After transfection, the cells were plated out in ProCHO4-complete medium in 96-well culture plates. After 24 h of growth a solution containing one or more selection agents were added (ProCHO4-complete selection medium; G418: 400 μg/ml; hygromycin: 600 μg/ml; puromycin: 8 μg/ml). Once a week the ProCHO4-complete selection medium was replaced. The antibody concentration of the anti-Aβ antibody was analyzed with an ELISA assay specific for human IgG1 in the culture supernatants.

For selection of high-yield anti-Aβ antibody production cell lines the productivity was tested in ProCHO4-complete selection medium after propagation in 6-well culture plates, T-flasks and/or Erlenmeyer shake flasks using an anti-human IgG1 ELISA and/or analytic Protein A HPLC.

Subclones were obtained by two methods, Limiting Dilution (LD) and Fluorescence Activated Cell Sorting (FACS).

Limiting Dilution:

For limiting dilution cells were plated out in ProCHO4-conditioned medium (consisting of 50% (v/v) fresh ProCHO4-complete selection medium and 50% (v/v) ProCHO4-complete conditioned selection medium derived from the cells to be propagated) at a cell density of 0.5-2 cells per 0.1 ml medium per well of a 96-well culture plate. Once a week the medium was replaced by ProCHO4-complete selection medium. The antibody concentration of the anti-Aβ antibody was analyzed by an ELISA assay specific for human IgG1 in the culture supernatants.

Single Cell Deposition by Flow Cytometry Including Identification and Isolation of Clones:

The identification and isolation of stably transfected clones was performed with the aid of a cell surface labeling technique using fluorescently tagged Protein A that binds to secreted but still membrane-attached antibodies. The fluorescence intensity of the stained cells was used as criterion for cell selection.

In the case of fluorescence activated cell sorting the electroporated population of cells were directly seeded into T-flasks in ProCHO4-complete medium. The appropriate selection agent or agents (G418, hygromycin, and/or puromycin) was/were added to the culture one day after transfection and the transfectant pool was expanded.

Cells from the expanded transfectant pool were first treated with Accumax (PAA Laboratories) for 15 minutes at 37° C. and then passed through a 40 μM nylon mesh to remove remaining large cell aggregates. The cells were collected by centrifugation, resuspended in PBS containing 5% FCS (Gibco/Invitrogen) at a cell density of 10^6 to 10^7 cells/ml and incubated for 20 minutes on ice. Thereafter, the cells were stained with 10 ng/ml Protein A Alexa Fluor 488 (Molecular Probes Inc.) in a volume of 8 ml FCS-PBS for 30 minutes on ice in the dark. Afterwards, the cells were washed once with 5% FCS-PBS and once with ProCHO4 medium containing 8 mM Ultra Glutamine (Cambrex Corp.), 1×HT supplement and 5% FCS. Finally the cells were resuspended in the supplemented ProCHO medium used for washing at a cell density of 10^6 to 10^7 cells/ml and transferred to a BD FACSAria cell sorter (BD Biosciences).

Single cells were sorted by flow cytometry and deposited in wells of 96-well culture plates containing of ProCHO4-conditioned medium. The selected and deposited cells encompassed cells with the top 10%, 7%, or 4% of fluorescence intensity of the gated live cells. After 48 hours ProCHO4 complete selection medium containing the appropriate selection agent in 2-fold concentration was added to each well. Once a week the medium was replaced with ProCHO4-complete selection medium. The antibody concentration of the anti-Aβ antibody was analyzed with an ELISA assay specific for human IgG1 in the culture supernatants.

Transfection and Selection Steps:

For the first transfection and selection step the plasmid 5137 has been used. Plasmid 5137 has been transfected with electroporation into parent cell line adapted to growth in ProCHO4-complete medium. The transfected cells were cultivated in ProCHO4-complete medium supplemented with up to 700 μg/ml G418 in 96 well plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. Approximately 1000 clones have been tested and the selected of them were further cultivated in 24-well plates, 6-well plates and subsequently in shaker flasks. The growth and productivity of approximately 20 clones was assessed in static and suspension cultures by anti-human IgG1 ELISA and/or analytic protein A HPLC. The best clone (best clone does not denote the most productive clone it denotes the clone with the best properties for the further steps) was subcloned by limited dilution in ProCHO4-conditioned medium supplemented with 700 μg/ml G418. The selected clone was named 8C8.

For the second transfection and selection step the plasmid 5128 has been used. Plasmid 5128 has been transfected with electroporation into cell line clone 8C8 cultivated in ProCHO4-complete medium supplemented with 700 μg/ml G418. The transfected cells were expanded for about two to three weeks in ProCHO4-conditioned medium supplemented with 200 μg/ml G418 and 300 μg/ml hygromycin (ProCHO4-double selection medium). Single antibody secreting cells were identified and deposited on the basis of their fluorescence intensity after staining with a Protein A Alexa Fluor conjugate by FACS analysis. The deposited cells were cultivated in ProCHO4-double selection medium in 96 well plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. Approximately 500 clones have been tested and the selected of them were further cultivated in 24-well plates, 6-well plates and subsequently in shaker flasks. The growth and productivity of approximately 14 clones was assessed in static and suspension cultures by anti-human IgG1 ELISA and/or analytic Protein A HPLC. The selected clone was named 4F5.

For the third transfection and selection step the plasmid 5151 has been used. Plasmid 5151 has been transfected with electroporation into cell line clone 4F5 cultivated in ProCHO4-double selection medium. The transfected cells were expanded for about two to three weeks in ProCHO4-triple selection medium (ProCHO4-conditioned medium supplemented with 200 μg/ml G418 and 300 μg/ml hygromycin and 4 μg/ml puromycin). Single antibody secreting cells were identified and deposited on the basis of their fluorescence intensity after staining with a Protein A Alexa Fluor conjugate by FACS analysis. The deposited cells were cultivated in ProCHO4-triple selection medium in 96 well plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. Approximately 500 clones have been tested and the selected of them were further cultivated in 24-well plates, 6-well plates and subsequently in shaker flasks. The growth and productivity of approximately 10 clones was assessed in

static and suspension cultures by anti-human IgG1 ELISA and/or analytic protein A HPLC. The selected clone was named 20F2.

Clone 20F2 has been selected based on his growth, productivity, and product quality characteristics after growth in fed-batch suspension culture in ProCHO4-triple selection medium, i.e. in the concomitant presence of the three selecting agents G418, hygromycin, and puromycin.

Clone Characteristics:

As can be seen from the following table the doubling time and cell density after three days of cultivation were comparable when the basic cell line CHO-K1 (wild-type) and the selected clones are compared.

TABLE 1

Growth characteristics

Clone	Doubling time [h]	Starting cell density [10^6 cells/ml]	Cell density at day 3 [10^6 cells/ml]	Viability at day 3 [%]
CHO-K1 (wild-type)	22-23	3	18-20	97-98
8C8	26-28	3	12-15	96-98
4F5	22-24	3	24-27	96-97
20F2	24-26	2	23-26	97-98

Example 3

Stability of Clone 20F2 Expressing an Anti-Aβ Antibody

Stability of growth and product formation was evaluated in sequential cell subculture over a time period of 60 days (about 60 generations) in the presence and absence of the selection agents (with and without antibiotics). The cultivation was performed as described above.

TABLE 2

Characteristics of clone 20F2.

Parameter	Clone 20F2: cultivation in the presence of three selection agents	Clone 20F2: cultivation in the absence of selection agents
Mean value viability [%]	97	97
Mean value doubling time [h]	27	26
Mean value SPR [pg/c/d]	11	9

Following extensive passage (up to generation 60) no evidence was obtained indicating that the anti-Aβ antibody producing clone 20F2 was unstable with respect to cell growth and product formation in the presence or absence of the three selection agents, respectively.

Example 4

Expression Vector for Expressing an Anti-P-Selectin Antibody

Another example (preferably monoclonal) antibody for which a cell line for expression can be obtained according to the current invention is an antibody against the human P-Selectin glycoprotein (anti-P-Selectin antibody). Such an antibody and the corresponding nucleic acid sequences are for example described in WO 2005/100402, or US 2005/0226876 or SEQ ID NO: 13 to 18.

The anti-P-Selectin antibody expressing Chinese hamster ovary cell line was generated by two successive complete transfections and clone selection campaigns.

A genomic human kappa-light chain constant region gene segment (C-kappa) was added to the light chain variable region of the anti-P-Selectin antibody, whereas a human gamma 4-heavy chain constant region gene segment (C_{H1}-Hinge-C_{H2}-C_{H3}) was added to the heavy chain variable region of the anti-P-Selectin antibody. The complete kappa-light and gamma 4-heavy chain antibody genes were then joined with a human cytomegalovirus immediate early promoter and enhancer (CMV IE) at the 5'-end and the Simian Virus 40 early polyadenylation (SV 40 early poly A) signal sequence at the 3'-end.

a) Heavy Chain Expression Cassette

The transcription unit of the anti-P-Selectin antibody heavy chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (CMV IE),
- a 5'-untranslated region (5' UTR),
- the coding sequence for the anti-P-Selectin antibody gamma 4-heavy chain including a signal peptide in an intron-exon gene structure,
- the SV 40 early poly A signal sequence.

b) Light Chain Expression Cassette

The transcription unit of the anti-P-Selectin antibody light chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (CMV IE),
- a 5'-untranslated region (5' UTR),
- the coding sequence for the anti-P-Selectin kappa-light chain in an intron-exon gene structure,
- the SV 40 early poly A signal sequence.

c) Expression Plasmids 5057 and 5069

For the expression and production of the anti-P-Selectin antibody the light and heavy chain expression cassettes were placed on a single expression vector (light chain upstream of heavy chain). Two identical expression vectors were generated differing only in the selectable marker gene included, in particular, the murine dihydrofolate reductase (DHFR) gene or a neomycin resistance gene.

The expression vectors contain beside the light and heavy chain expression cassette the following elements:

- a selectable marker, either the murine DHFR gene or a gene conferring resistance to the selection agent neomycin under the control of the SV40 early promoter and origin,
- an origin of replication allowing for the replication of the plasmid in *E. coli* taken from pUC19 (pUC origin),
- a beta-lactamase gene which confers ampicillin resistance in *E. coli*.

The plasmid map of the expression vector 5057 containing the murine DHFR marker gene is shown in FIG. **4**. The plasmid map of the expression vector 5069 containing a neomycin selectable marker gene is shown in FIG. **5**.

Example 5

Transfection and Selection of a CHO Cell Line Expressing an Anti-P-Selectin Antibody

CHO-K1 cells, pre-adapted to growth in serum-free suspension culture in protein-free HyQ SFM4CHO medium (Hyclone, Cat. No. SH30549) supplemented with defined animal-derived components (cholesterol from ovine wool

and cod-liver oil) were used as the host cell line. The cells were propagated in shake flasks in protein-free HyQ SFM4CHO medium under standard humidified conditions (95%, 37° C., and 5% CO_2) and under constant agitation at 150 rpm/min. Depending on the cell density the cells were split into fresh medium.

The adherent CHO-K1 cell lines had been obtained from the American Type Culture Collection as ATCC CCL-61.

First Transfection and Selection

Prior to transfection the expression plasmid 5057 was linearized within the beta-lactamase gene using the restriction enzyme PvuI. The cleaved DNA was purified using QiaQuick spin columns (Qiagen) according to the manufacturer's recommendations.

Transfection was carried out by electroporation using Gene Pulser XCell (BIO-RAD) and 0.2 cm-cuvettes (BIO-RAD, Cat. No. 165-2086). For transfection 10^6 to 10^7 CHO-K1 cells were harvested by centrifugation, resuspended in PBS, transferred to the cuvette and mixed with 20-50 μg linearized plasmid DNA. The cells were exposed to a single square wave pulse (160 V, 15 ms) and subsequently diluted in HyQ SFM4CHO medium to a density of approx. 4×10^5 cells/ml and seeded in a T75 cell culture flask. After 48 hours of propagation without the supplementation of a selection agent, the cells were diluted in HyQ SFM4CHO medium supplemented with 200 nM MTX to a density of 10^4 to 10^5 cells/ml and seeded in 96-well plates with 3-7000 cells per well. After approx. two weeks, fresh medium was added per well and after additional two weeks the culture medium was completely replaced by fresh medium. Four days later the culture supernatants were tested for antibody production by anti-human Fc ELISA. In total approximately 600 clones were screened.

45 clones with antibody titers of more than 10 μg/ml were picked and transferred to 48-well plates. The clones were expanded to shaker flasks over additional passages and subsequently transferred to serum free production medium for the final productivity assessment. A 125 ml shaker flask was inoculated with 10^5 to 10^6 cells/ml in medium supplemented with 200 nM MTX. Viable cell density and viability were monitored over one week. Antibody titers were measured by Protein A chromatography on the final day. Based on these data, clone G24 was selected for further development. G24 reached a maximal viable cell density of 3.3×10^6 cells/ml. The antibody titer was 402 μg/ml. The average specific production rate (SPR) was 28 pg/(cell*d).

Second Transfection and Selection:

Clone G24 was subjected to a second transfection. For the second transfection plasmid 5069 was used. Linearization and purification of the plasmid as well as electroporation of G24 were performed as described for the first transfection. After 48 hours of propagation without selection pressure, the cells were diluted in HyQ SFM4CHO medium supplemented with 200 nM MTX and 400 μg/ml G418 to a density of 10^3 to 10^4 cells/ml and seeded in 96-well plates with 500 cells per well. After approx. two weeks, fresh medium was added per well and after an additional week the culture medium was completely replaced by fresh medium. Four days later the culture supernatants were tested for antibody production by anti-human Fc ELISA. In total approximately 220 clones were screened.

Then 13 clones with antibody titers of more than 150 μg/ml were picked and transferred to 24-well plates. The clones were expanded to shaker flasks over additional passages and subsequently transferred to serum free production medium for the final productivity assessment. A shaker flask was inoculated with 10^5 to 10^6 cells/ml in 50 ml medium supplemented with 200 nM MTX and 400 μg/ml G418. Viable cell density and viability were monitored over one week, Antibody titers were measured by Protein A chromatography on the final day. Based on these data, clone G24_x6 was considered the best clone. G24_x6 reached a maximal viable cell density of 3.0×10^6 cells/ml. The antibody titer was 685 μg/ml. The average specific production rate (SPR) from was 48 pg/(cell*d).

Limiting Dilution:

To compare the method according to the invention with simple subcloning with respect to their effect on productivity we subjected clone G24 to limited dilution or single cell deposition in 96-well plates.

For limiting dilution the cells were seeded in 96-well plates in HyQ SFM4CHO medium supplemented with 50% (v/v) conditioned medium, 10% FCS and 200 nM MTX at 0.5 cells/well. Alternatively 1 cell/well was deposited in 96-well plates by FACS. After 10 days, fresh HyQ SFM4CHO medium, 200 nM MTX without FCS was added per well and after an additional week the culture medium was completely replaced by HyQ SFM4CHO medium, 200 nM MTX. Four days later the culture supernatants were tested for antibody production by anti-human Fc ELISA. In total approximately 230 clones were screened.

Eleven subclones with antibody titers of more than 130 μg/ml were transferred to 24-well plates. After passages in 6-well plates, the clones were transferred to shaker flasks and subsequently transferred to serum free production medium for the final productivity assessment. A shaker flask was inoculated with 10^5 to 10^6 cells/ml in medium supplemented with 200 nM MTX. Viable cell density and viability were monitored over one week. Antibody titers were measured by Protein A chromatography on the final day. Based on these data G24_13 was considered the best clone. G24_13 reached a maximal viable cell density of 3.6×10^6 cells/ml. The antibody titer was 472 μg/ml. The average the specific production rate (SPR) was 31 pg/(cell*d).

Table 3 summarizes the productivity data of best performing subclone G24_13 and the best performing clone G24_x6 obtained with the method according to the invention in comparison to their parental clone G24. With the method according to the invention a clone with volumetric and specific productivity increased by more than 50% can be obtained whereas after subcloning only a minor increase of both parameters was observed.

FIG. **6** shows an overview of the volumetric (A) and specific (B) productivities of all subclones of G24 that had been investigated in shake flasks. As can be seen, the average volumetric and specific productivity of the clones obtained with the method according to the invention was significantly higher than after subcloning.

TABLE 3

Productivity of the best producing clones compared to the parental clone G24.

	G24	G24_13 (Subclone)	G24_x6 (method according to the invention)
Antibody concentration in the supernatant [μg/ml]	402	472	685
SPR pg/(cell * d)]	28	31	48
Max. cell density [10^5/ml]	33	36	30

Clone Characteristics:

As can be seen from the following table the doubling time and the cell density after three days of cultivation were comparable when the one-time transfected cell line G24 and the selected clones are compared.

TABLE 4

Growth characteristics				
Clone	Doubling time [h]	Starting cell density [10^6 cells/ml]	Cell density at day 3 [10^6 cells/ml]	Viability at day 3 [%]
G24	29	0.3	0.7	91
G24_13	27	0.3	2.0	91
G24_x6	24	0.3	2.5	93

Example 6

Transfection and Selection of a CHO Cell Line Expressing an Anti-P-Selectin Antibody

CHO-DG44 cells pre-adapted to growth in serum-free suspension culture in protein-free HyQ SFM4CHO medium (Hyclone, Cat. No. SH30549) were used as the host cell line. The host cell line was cultured in commercial medium HyQ SFM4CHO-utility (Hyclone, Cat. No. SH30516) during transfections, screening and subcloning steps.

First Transfection and Selection

Prior to transfection the expression plasmid 5057 (FIG. **4**) was linearized within the beta-lactamase gene using the restriction enzyme PvuI.

The transfection of the host cell line was performed by nucleotransfection provided by AMAXA (Nucleofector Kit T, Cat. No. VCA-1002, Transfection program U-17). Cells were cultured in medium supplemented with 10% fetal calf serum for 48 h after transfection.

Transfected cells were plated on 96-well plates with 1000 cells per well in medium supplemented with 10% fetal calf serum in the presence of 40 nM methotrexate (MTX) as selection agent and incubated for approx. three weeks.

Antibody concentration was determined by ELISA in the supernatant of the 96-well plates. About 400 primary clones were screened. Twenty-four clones with the highest antibody productivity were transferred to 24-well plates and cultivated in the presence of the selection agent without supplementation with fetal calf serum.

Product quality was analyzed by Western Blotting detecting light and heavy antibody chains. Nine clones which showed the highest productivity and which expressed antibody without detectable antibody derived side products (Western blot) were expanded into shake flasks.

Productivity was analyzed in batch shake flasks after 7 and 10 days of incubation. Product quality was assessed by SDS-PAGE after Protein-A HPLC purification (FIG. **7**). Best product concentration was reached with clone 43-16. Best specific productivity per cell was achieved with clone 35-45. Both clones showed no detectable side products in the SDS-PAGE. Both clones were selected for subcloning by limiting dilution.

Parental clones 35-45 and 43-16 were subcloned by limiting dilution on 96-well plates in commercial HyQ medium supplemented with 5% (v/v) fetal calf serum in the presence of 20 nM MTX. After 20 days of incubation antibody production was screened by ELISA. Best subclones in terms of productivity were expanded to shake flasks and subsequently transferred to serum free production medium for the final productivity assessment. The two best subclones, 35-45-F2 and 43-16-A10, of the parental clones 35-45 and 43-16 were assessed in standard batch shake flask assay. Productivity was 270 μg/ml and 185 μg/ml after 7 days and 337 μg/ml and 343 μg/ml after 10 days, respectively.

Second Transfection and Selection:

Subclone 43-16-A10 was transfected with the expression vector p5069 (FIG. **5**) using the nucleofection method (Amaxa Nucleofector Kit T, VCA-1002, Transfection program U-17). The second transfection was also carried out in Hyclone medium: HyQ SFM4CHO-utility (Cat. No. SH30516) supplemented with 10% fetal calf serum and 20 nM MTX. Two days after the second transfection cells were transferred to 96-well plates with 1000 cells per well. As second selection agent 250 μg/ml G418 was added.

After cultivation for two weeks more than 2000 primary wells were screened by antibody titer determination by anti-human Pc ELISA. Fifty clones with highest productivity were transferred into 24-well plates and screened a second time by anti-human Fc ELISA three days later. All clones were transferred to 6-well plates and screened by anti-human Pc ELISA three days later. The six clones with the best productivity were directly subcloned from the 6-well plate stage.

Limiting Dilution:

The best parental clones of the second transfection and selection round 43-16A10-S1, 43-16A10-S13, 43-16A10-S14, 43-16A10-S19, 43-16A10-S24, 43-16A10-S43 were subcloned by limiting dilution. The product quality of the twelve best subclones was assessed in SDS-PAGE and Western-Blotting from the 24-well stage. No unwanted antibody related side products were detected.

Three subclones, 43-16-A10-S1-16, 43-16-A10-S24-11, and 43-16-A10-S43-14, were selected according to their productivity in 6-well plates for the expansion in shake flasks. They were transferred to serum free production medium for the final productivity assessment. Their productivity was compared to the subclone after the first transfection, clone 43-16-A10. The productivity was increased two-fold for two of the clones after the second transfection and selection, 43-16-A10-S1-16 and 43-16-A10-S24-11, from 221 μg/ml after 7 days in the batch shake flask to 436 μg/ml and 407 μg/ml, respectively. After 10 days incubation in the batch shake flask the productivity increased from 306 μg/ml to 683 μg/ml and 446 μg/ml, respectively.

The specific productivity per cell increased as well from 17 pg/cell/day for the clone 43-16-A10 after the first transfection to 40 pg/cell/day for the first transfected clone 43-16-A10-S1-16 and to 33 pg/cell/day for the second transfected clone 43-16-A10-S24-11. The doubling time was not affected by the second transfection. The doubling time for the clone 43-16-A10 after the first transfection was 33 h and it was 32 h for both clones 43-16-A10-S1-16 and 43-16-A10-S24-11.

Example 7

Expression Vector for Expressing an Anti-IL-13Rα Antibody

Another example (preferably monoclonal) antibody for which a cell line for expression can be obtained according to the current invention is an antibody binding to the IL-13 Receptor alpha I (anti-IL-13Rα1 anti-IL-13Rα antibody).

Such an antibody and the corresponding nucleic acid sequences are for example described in WO 2006/072564 or SEQ ID NO: 19 to 28.

A genomic human kappa-light chain constant region gene segment (C-kappa) was added to the light chain variable region of the anti-IL-13Rα antibody whereas a human gamma 1-heavy chain constant region gene segment (C_{H1}-Hinge-C_{H2}-C_{H3}) was added to the heavy chain variable region of the anti-IL-13Rα antibody. The expression plasmid 5321 comprises an expression cassette for the anti-IL-13Rα antibody γ1-heavy chain, and the anti-IL-13Rα antibody κ-light chain, and a nucleic acid encoding the murine DHFR gene. An annotated plasmid map is shown in FIG. **9**.

a) Heavy Chain Expression Cassette

The transcription unit of the anti-IL-13Rα antibody conjugate heavy chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (CMV IE),
- a 5'-untranslated region (5' UTR),
- the coding sequence for the anti-IL-13Rα antibody gamma 1-heavy chain conjugate including a signal peptide in an intron-exon gene structure,
- the human gamma 1-immunoglobulin polyadenylation signal sequence.

b) Light Chain Expression Cassette

The transcription unit of the anti-IL-13Rα antibody light chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (CMV IE),
- a 5'-untranslated region (5' UTR),
- the coding sequence for the anti-IL-13Rα kappa-light chain in an intron-exon gene structure,
- the human immunoglobulin kappa-polyadenylation signal sequence.

c) Expression Plasmids

For the expression and production of the anti-IL-13Rα antibody conjugate the light and heavy chain expression cassettes were placed on a single expression vector (light chain upstream of heavy chain). Two identical expression vectors were generated differing only in the selectable marker gene included, in particular, the murine DHFR gene and both the murine DHFR gene and a hygromycin resistance gene.

The expression vectors contain beside the light and heavy chain expression cassette the following elements:

- an origin of replication allowing for the replication of the plasmid in *E. coli* (pUC origin),
- a beta-lactamase gene which confers ampicillin resistance in *E. coli*.

Example 8

Transfection and Selection of a CHO Cell Line Expressing an Anti-IL-13Rα Antibody

For the first transfection and selection step the plasmid 5321 has been used. Plasmid 5321 has been transfected with electroporation into parent cell line adapted to growth in ProCHO4-complete medium. The transfected cells were cultivated in HyQSFMCHO-medium (HyClone) supplemented with up to 200 nM methotrexate in plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. The clones have been tested and the selected of them were further cultivated in 24-well plates, 6-well plates and subsequently in shaker flasks. The growth and productivity was assessed in static and suspension cultures by anti-human IgG1 ELISA and/or analytic Protein A HPLC. The best clone (best clone does not denote the most productive clone it denotes the clone with the best properties for the further steps) was selected. The selected clone was named 200_019. Productivity was 90 μg/ml with an average specific production rate of 7 pg/cell*d after 7 days of cultivation.

For the second transfection and selection step a plasmid with a DHFR and hygromycin resistance gene has been used. The plasmid has been transfected with electroporation into the selected cell line cultivated in HyQSFMCHO-medium (HyClone) supplemented with up to 200 nM methotrexate. The double selection medium contained in addition 300 μg/ml hygromycine B. Single antibody secreting cells were identified and deposited on the basis of their fluorescence intensity after staining with a Protein A Alexa Fluor conjugate by FACS analysis. The selected clone was named 5_17_35. Productivity was 150 μg/ml with an average specific production rate of 10 pg/cell*d after 7 days of cultivation.

Example 9

Expression Vector for Expressing an Anti-CD4 Antibody Conjugate

Another example (monoclonal) antibody for which a cell line for expression can be obtained according to the current invention is an antibody against the human CD4 surface receptor (anti-CD4 antibody) which is conjugated to two to eight antifusogenic peptides. Such an antibody and the corresponding nucleic acid sequences are for example reported in PCT/EP2008/005894 or SEQ ID NO: 29 to 40.

A genomic human kappa-light chain constant region gene segment (C-kappa) was added to the light chain variable region of the anti-CD4 antibody of SEQ ID NO: 39, whereas a human gamma 1-heavy chain constant region gene segment (C_{H1}-Hinge-C_{H2}-C_{H3}) was added to the heavy chain variable region of the anti-CD4 antibody of SEQ ID NO: 36. The expression plasmid 6311 comprises an anti-CD4 antibody γ1-heavy chain, which is joint at the last but one C-terminal amino acid, i.e. the C-terminal lysine residue of the heavy chain is removed, with a nucleic acid encoding an antifusogenic peptide of SEQ ID NO: 41 via the peptidic glycine-serine linker of SEQ ID NO: 42, and a anti-CD4 antibody κ-light chain, and a nucleic acid conferring resistance to the selectable marker neomycin. An annotated plasmid map is shown in FIG. **8**.

a) Heavy Chain Expression Cassette

The transcription unit of the anti-CD4 antibody conjugate heavy chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (CMV IE),
- a 5'-untranslated region (5' UTR),
- the coding sequence for the anti-CD4 antibody gamma 1-heavy chain conjugate including a signal peptide in an intron-exon gene structure,
- the SV 40 early poly A signal sequence.

b) Light Chain Expression Cassette

The transcription unit of the anti-CD4 antibody conjugate light chain is composed of the following elements:

- the immediate early enhancer and promoter from the human cytomegalovirus (CMV IE),
- a 5'-untranslated region (5' UTR),

the coding sequence for the anti-CD4 kappa-light chain in an intron-exon gene structure,

the SV 40 early poly A signal sequence.

c) Expression Plasmids

For the expression and production of the anti-CD4 antibody conjugate the light and heavy chain expression cassettes were placed on a single expression vector (light chain upstream of heavy chain). Three identical expression vectors were generated differing only in the selectable marker gene included, in particular, a neomycin resistance gene, a puromycin resistance gene, and a hygromycin resistance gene.

The expression vectors contain beside the light and heavy chain expression cassette the following elements:

an origin of replication allowing for the replication of the plasmid in *E. coli* taken from pUC18 (pUC origin),

a beta-lactamase gene which confers ampicillin resistance in *E. coli*.

Example 10

Transfection and Selection of a CHO Cell Line Expressing an Anti-CD4 Antibody Conjugate

Transfection and Selection Steps:

For the first transfection and selection step the plasmid 6311 has been used. Plasmid 6311 has been transfected with electroporation into parent cell line adapted to growth in ProCHO4-complete medium. The transfected cells were cultivated in ProCHO4-complete medium supplemented with up to 700 μg/ml G418 in 96 well plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. Approximately 5000 clones have been tested and the selected of them were further cultivated in 24-well plates, 6-well plates and subsequently in shaker flasks. The growth and productivity of approximately 15 clones was assessed in static and suspension cultures by anti-human IgG1 ELISA and/or analytic Protein A HPLC. The best clone (best clone does not denote the most productive clone it denotes the clone with the best properties for the further steps) was subcloned by limited dilution in ProCHO4-conditioned medium supplemented with 700 μg/ml G418.

Subclones were obtained by two methods, Limiting Dilution (LD) and Fluorescence Activated Cell Sorting (FACS).

Limiting Dilution:

For limiting dilution cells were plated out in ProCHO4-selection medium at a cell density of 0.5-2 cells per 0.1 ml medium per well of a 96-well culture plate.

Single Cell Deposition by Flow Cytometry Including Identification and Isolation of Clones:

In the case of fluorescence activated cell sorting the electroporated population of cells were directly seeded into T-flasks in ProCHO4-complete medium. The appropriate selection agent or agents (G418, hygromycin, and/or puromycin) was/were added to the culture one day after transfection and the transfectant pool was expanded. The growth and productivity of approximately 112 clones was assessed in static and suspension cultures by anti-human IgG1 ELISA and/or analytic Protein A HPLC. The selected clone was named I-17.

For the second transfection and selection step a plasmid with a hygromycin resistance gene has been used. The plasmid has been transfected with electroporation into cell line clone I-17 cultivated in ProCHO4-complete medium supplemented with 700 μg/ml G418. The transfected cells were expanded for about two to three weeks in ProCHO4-conditioned medium supplemented with 200 μg/ml G418 and 300 μg/ml hygromycin (ProCHO4-double selection medium). Single antibody secreting cells were identified and deposited on the basis of their fluorescence intensity after staining with a Protein A Alexa Fluor conjugate by FACS analysis. The deposited cells were cultivated in ProCHO4-double selection medium in 96 well plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. The selected clone was named 24_16.

For the third transfection and selection step a plasmid with a puromycin resistance gene has been used. The plasmid has been transfected with electroporation into cell line clone 24_16 cultivated in ProCHO4-double selection medium. The transfected cells were expanded for about two to three weeks in ProCHO4-triple selection medium (ProCHO4-conditioned medium supplemented with 200 μg/ml G418 and 300 μg/ml hygromycin and 4 μg/ml puromycin). Single antibody secreting cells were identified and deposited on the basis of their fluorescence intensity after staining with a Protein A Alexa Fluor conjugate by FACS analysis. The deposited cells were cultivated in ProCHO4-triple selection medium in 96 well plates. The antibody concentration in the culture supernatants was evaluated by an anti-human IgG1 ELISA. The selected clone was named 1_24.

Clone Characteristics:

As can be seen from the following table the doubling time and the cell density after three days of cultivation were comparable when the basic cell line CHO-K1 (wild-type) and the selected clones are compared.

TABLE 5

Growth characteristics

Clone	Doubling time [h]	Starting cell density [10^6 cells/ml]	Cell density at day 3 [10^6 cells/ml]	Viability at day 3 [%]
CHO-K1 (pre adapted)	22-25	3	18-22	96-98
I-17	25-30	3	13-15	95-97
24_16	25-30	3	15-16	95-96
1_24	30-32	3	12-14	95-97

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    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

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            20                  25                  30

Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ser Ala Ile Ser Gly Ser Gly Gly Ser Thr Tyr Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

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        35                  40                  45

Ser Ala Ile Ser Gly Ser Gly Gly Ser Thr Tyr Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Leu Leu Ser Arg Gly Tyr Asn Gly Tyr Tyr His Lys Phe Asp
            100                 105                 110

Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 10
<211> LENGTH: 110
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: VL

<400> SEQUENCE: 10

Asp Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly
1               5                   10                  15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Ser
            20                  25                  30

Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu
        35                  40                  45

Ile Tyr Gly Ala Ser Ser Arg Ala Thr Gly Val Pro Ala Arg Phe Ser
    50                  55                  60

Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu
65                  70                  75                  80

Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Val Tyr Asn Pro Pro
                85                  90                  95

Val Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
            100                 105                 110


<210> SEQ ID NO 11
<211> LENGTH: 110
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: VL

<400> SEQUENCE: 11

Asp Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly
1               5                   10                  15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Ser
            20                  25                  30

Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu
        35                  40                  45

Ile Tyr Gly Ala Ser Ser Arg Ala Thr Gly Val Pro Ala Arg Phe Ser
    50                  55                  60

Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu
65                  70                  75                  80

Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Phe Gln Leu Tyr Ser Asp Pro
                85                  90                  95

Phe Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
            100                 105                 110
```

```
<210> SEQ ID NO 12
<211> LENGTH: 110
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: VL

<400> SEQUENCE: 12

Asp Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly
1               5                   10                  15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Ser
            20                  25                  30

Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu
        35                  40                  45

Ile Tyr Gly Ala Ser Ser Arg Ala Thr Gly Val Pro Ala Arg Phe Ser
    50                  55                  60

Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu
65                  70                  75                  80

Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Leu Ser Ser Phe Pro
                85                  90                  95

Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
            100                 105                 110


<210> SEQ ID NO 13
<211> LENGTH: 124
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 13

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr
            20                  25                  30

Asp Met His Trp Val Arg Gln Ala Thr Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ser Gly Ile Thr Thr Ala Gly Asp Thr Tyr Tyr Pro Gly Ser Val Lys
    50                  55                  60

Gly Arg Phe Thr Ile Ser Arg Glu Asn Ala Lys Asn Ser Leu Tyr Leu
65                  70                  75                  80

Gln Met Asn Ser Leu Arg Ala Gly Asp Thr Ala Val Tyr Tyr Cys Ala
                85                  90                  95

Arg Gly Arg Ile Ser Met Asp Arg Gly Val Lys Asn Asn Trp Phe Asp
            100                 105                 110

Pro Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 14
<211> LENGTH: 124
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 14

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Arg Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asn Tyr
            20                  25                  30

Asp Met His Trp Val Arg Gln Ala Thr Gly Lys Gly Leu Glu Trp Val
        35                  40                  45
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```
Ser Ala Ile Thr Ala Ala Gly Asp Ile Tyr Tyr Pro Gly Ser Val Lys
    50                  55                  60

Gly Arg Phe Thr Ile Ser Arg Glu Asn Ala Lys Asn Ser Leu Tyr Leu
65                  70                  75                  80

Gln Met Asn Ser Leu Arg Ala Gly Asp Thr Ala Val Tyr Tyr Cys Ala
                85                  90                  95

Arg Gly Arg Tyr Ser Gly Ser Gly Ser Tyr Tyr Asn Asp Trp Phe Asp
            100                 105                 110

Pro Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 15
<211> LENGTH: 124
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 15

Gln Pro Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ala
1               5                   10                  15

Ser Val Lys Val Ser Cys Lys Val Ser Gly Asn Thr Leu Thr Glu Leu
            20                  25                  30

Ser Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Met
        35                  40                  45

Gly Gly Phe Asp Pro Glu Asn Gly Glu Ala Ile Tyr Ala Gln Lys Phe
    50                  55                  60

Gln Gly Arg Val Thr Met Thr Ala Asp Thr Ser Thr Asp Thr Ala Tyr
65                  70                  75                  80

Met Asp Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Thr Asp Leu Ala Gly Gly Ser Asp Phe Tyr Tyr Tyr Gly Leu Asp
            100                 105                 110

Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 16
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 16

Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly
1               5                   10                  15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Tyr
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
        35                  40                  45

Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
65                  70                  75                  80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Asn Asn Trp Pro Leu
                85                  90                  95

Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
            100                 105


<210> SEQ ID NO 17
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<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 17

Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly
1               5                   10                  15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Tyr
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile
        35                  40                  45

Tyr Asp Ala Ser Asn Arg Ala Thr Gly Ile Pro Ala Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro
65                  70                  75                  80

Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Arg Ser Asn Trp Pro Leu
                85                  90                  95

Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
            100                 105


<210> SEQ ID NO 18
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 18

Ala Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Ser Ser Ala
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Asp Ala Ser Ser Leu Glu Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Phe Asn Ser Tyr Pro Tyr
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
            100                 105


<210> SEQ ID NO 19
<211> LENGTH: 119
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: LC5002-002 VH gamma/heavy chain variable domain

<400> SEQUENCE: 19

Glu Val Gln Leu Leu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Asn Ile Tyr
            20                  25                  30

Ala Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ser Val Ile Ser Gly Arg Gly Ile Thr Thr Tyr Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
```

```
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Asp Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Lys Gly Ser Ser Ser Trp Thr Asp Phe Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Leu Val Thr Val Ser Ser
        115


<210> SEQ ID NO 20
<211> LENGTH: 119
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: LC5002-003 VH gamma/heavy chain variable domain

<400> SEQUENCE: 20

Glu Val Gln Leu Leu Glu Ser Gly Gly Asp Leu Ile Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Asn Ile Tyr
            20                  25                  30

Ala Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ser Val Ile Ser Gly Arg Gly Ile Thr Thr Tyr Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asp Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Lys Gly Ser Ser Tyr Trp Thr Asp Phe Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Leu Val Thr Val Ser Ser
        115


<210> SEQ ID NO 21
<211> LENGTH: 118
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: LC5002-005 VH gamma/heavy chain variable domain

<400> SEQUENCE: 21

Glu Val Gln Val Leu Asp Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Thr Ala Ser Gly Phe Thr Phe Arg Leu Tyr
            20                  25                  30

Thr Met Ser Trp Val Arg Gln Thr Pro Gly Arg Gly Leu Glu Trp Val
        35                  40                  45

Ser Gly Ile Ser Gly Ser Gly Leu Ser Thr Tyr Phe Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Val Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Lys Glu Gly Asp Trp Ile Tyr Phe Asp Ser Trp Gly Gln Gly Thr
            100                 105                 110

Leu Val Ile Val Ser Ser
        115
```

<210> SEQ ID NO 22
<211> LENGTH: 123
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: LC5002-007 VH gamma/heavy chain variable domain

<400> SEQUENCE: 22

```
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Ser
1               5                   10                  15

Ser Val Lys Val Ser Cys Lys Val Ser Gly Gly Thr Phe Ser Ser Tyr
            20                  25                  30

Ala Phe Ser Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Met
        35                  40                  45

Gly Arg Ile Ile Pro Ile Leu Gly Arg Thr Asn Tyr Ala Gln Lys Phe
    50                  55                  60

Gln Gly Arg Val Thr Ile Thr Ala Asp Lys Ser Thr Ser Thr Ala Tyr
65                  70                  75                  80

Met Glu Val Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Glu Gly Glu Thr Leu Asp Tyr Phe Tyr Tyr Gly Met Asp Val
            100                 105                 110

Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
        115                 120
```

<210> SEQ ID NO 23
<211> LENGTH: 119
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: LC5002-018 VH gamma/heavy chain variable domain

<400> SEQUENCE: 23

```
Glu Val Gln Leu Leu Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Asn Ile Tyr
            20                  25                  30

Ala Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ser Val Ile Ser Gly Ser Gly Val Thr Thr Tyr Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Lys Gly Ser Ser Trp Tyr Val Asp Phe Asp Tyr Trp Gly Gln Gly
            100                 105                 110

Thr Leu Val Thr Val Ser Ser
        115
```

<210> SEQ ID NO 24
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: LC5002-002 VL kappa light/chain variable domain

<400> SEQUENCE: 24

```
Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
```

```
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Ser Arg Trp
            20                  25                  30

Val Ala Trp Tyr Gln Gln Lys Pro Glu Lys Ala Pro Lys Ser Leu Ile
        35                  40                  45

Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ser Tyr Pro Trp
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105


<210> SEQ ID NO 25
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: LC5002-003 VL kappa/light chain variable domain

<400> SEQUENCE: 25

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Ser Ser Trp
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys Ala Pro Lys Ser Leu Ile
        35                  40                  45

Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ser Tyr Pro Trp
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105


<210> SEQ ID NO 26
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: LC5002-005 VL kappa/light chain variable domain

<400> SEQUENCE: 26

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Ser Ser Trp
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys Ala Pro Lys Ser Leu Ile
        35                  40                  45

Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ser His Pro Pro
                85                  90                  95
```

```
Thr Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
            100                 105


<210> SEQ ID NO 27
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: LC5002-007 VL kappa/light chain variable domain

<400> SEQUENCE: 27

Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly
1               5                   10                  15

Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Ser
            20                  25                  30

Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu
        35                  40                  45

Ile Tyr Gly Ala Ser Ser Arg Ala Ile Gly Ile Pro Asp Arg Phe Ser
    50                  55                  60

Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg Leu Glu
65                  70                  75                  80

Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln His Tyr Gly Ser Ser Leu
                85                  90                  95

Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
            100                 105


<210> SEQ ID NO 28
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: LC5002-018 VL kappa/light chain variable domain

<400> SEQUENCE: 28

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Gly Ile Ser Ser Trp
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Glu Lys Ala Pro Lys Ser Leu Ile
        35                  40                  45

Tyr Ala Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Asn Ser Tyr Pro Trp
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105


<210> SEQ ID NO 29
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 29

Glu Lys Asp Asn Tyr Ala Thr Gly Ala Trp Phe Ala Tyr
1               5                   10


<210> SEQ ID NO 30
<211> LENGTH: 13
```

```
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 30

Glu Lys Asp Asn Tyr Ala Thr Gly Ala Trp Phe Ala Tyr
1               5                   10


<210> SEQ ID NO 31
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 31

Ala Arg Lys Tyr Gly Gly Asp Tyr Asp Pro Phe
1               5                   10


<210> SEQ ID NO 32
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 32

Gln Gln Tyr Tyr Ser Tyr Arg Thr
1               5


<210> SEQ ID NO 33
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 33

Gln Gln Tyr Tyr Ser Tyr Arg Thr
1               5


<210> SEQ ID NO 34
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 34

Tyr Asp Asn Leu Leu Phe
1               5


<210> SEQ ID NO 35
<211> LENGTH: 122
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: mutated heavy chain variable domain

<400> SEQUENCE: 35

Glu Val Lys Leu Gln Glu Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1               5                   10                  15

Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Val Ile His Trp Val Arg Gln Lys Pro Gly Gln Gly Leu Asp Trp Ile
        35                  40                  45

Gly Tyr Ile Asn Pro Tyr Asn Asp Gly Thr Asp Tyr Asp Glu Lys Phe
    50                  55                  60

Lys Gly Lys Ala Thr Leu Thr Ser Asp Lys Ser Ser Ser Thr Ala Tyr
65                  70                  75                  80

Met Glu Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                85                  90                  95
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Ala Arg Glu Lys Asp Asn Tyr Ala Thr Gly Ala Trp Phe Ala Tyr Trp
100 105 110

Gly Gln Gly Thr Thr Val Thr Val Ser Ser
115 120

<210> SEQ ID NO 36
<211> LENGTH: 122
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: mutated heavy chain variable domain

<400> SEQUENCE: 36

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Val Val Lys Pro Gly Ala
1 5 10 15

Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Val Ile His Trp Val Arg Gln Lys Pro Gly Gln Gly Leu Asp Trp Ile
35 40 45

Gly Tyr Ile Asn Pro Tyr Asn Asp Gly Thr Asp Tyr Asp Glu Lys Phe
50 55 60

Lys Gly Lys Ala Thr Leu Thr Ser Asp Thr Ser Thr Ser Thr Ala Tyr
65 70 75 80

Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Glu Lys Asp Asn Tyr Ala Thr Gly Ala Trp Phe Ala Tyr Trp
100 105 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

<210> SEQ ID NO 37
<211> LENGTH: 120
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: mutated heavy chain variable domain

<400> SEQUENCE: 37

Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg
1 5 10 15

Ser Leu Arg Leu Ser Cys Ser Ala Ser Gly Phe Thr Phe Ser Asn Tyr
20 25 30

Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Ala Ile Ser Asp His Ser Thr Asn Thr Tyr Tyr Pro Asp Ser Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Phe
65 70 75 80

Leu Gln Met Asp Ser Leu Arg Pro Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Lys Tyr Gly Gly Asp Tyr Asp Pro Phe Asp Tyr Trp Gly Gln
100 105 110

Gly Thr Pro Val Thr Val Ser Ser
115 120

<210> SEQ ID NO 38
<211> LENGTH: 112
<212> TYPE: PRT

```
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: mutated light chain variable domain

<400> SEQUENCE: 38

Asp Ile Val Met Thr Gln Ser Pro Ser Ser Leu Ala Val Ser Val Gly
1               5                   10                  15

Glu Lys Val Thr Met Ile Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser
            20                  25                  30

Thr Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
        35                  40                  45

Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser Gly Val
    50                  55                  60

Pro Asp Arg Phe Thr Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
65                  70                  75                  80

Ile Ser Ser Val Lys Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
                85                  90                  95

Tyr Tyr Ser Tyr Arg Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110


<210> SEQ ID NO 39
<211> LENGTH: 112
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: mutated light chain variable domain

<400> SEQUENCE: 39

Asp Ile Val Met Thr Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly
1               5                   10                  15

Glu Arg Val Thr Met Asn Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser
            20                  25                  30

Thr Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln
        35                  40                  45

Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Thr Arg Glu Ser Gly Val
    50                  55                  60

Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr
65                  70                  75                  80

Ile Ser Ser Val Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Gln
                85                  90                  95

Tyr Tyr Ser Tyr Arg Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105                 110


<210> SEQ ID NO 40
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: mutated light chain variable domain

<400> SEQUENCE: 40

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Asn Asn Tyr
            20                  25                  30

Ile Ala Trp Tyr Gln His Thr Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

His Tyr Thr Ser Thr Leu Gln Pro Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60
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Ser Gly Ser Gly Thr Asp Tyr Thr Phe Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Ile Ala Thr Tyr Cys Leu Gln Gln Tyr Asp Asn Leu Leu Phe
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Leu Gln Ile Thr Arg
            100                 105


<210> SEQ ID NO 41
<211> LENGTH: 37
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: afp-1

<400> SEQUENCE: 41

Asn Met Thr Trp Met Glu Trp Asp Arg Glu Ile Asn Gln Tyr Thr Ser
1               5                   10                  15

Leu Ile His Ser Leu Ile Glu Glu Ser Gln Asn Gln Gln Glu Lys Asn
            20                  25                  30

Glu Gln Glu Leu Leu
        35


<210> SEQ ID NO 42
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: linker

<400> SEQUENCE: 42

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1               5                   10                  15
```

The invention claimed is:

1. A CHO cell secreting a heterologous immunoglobulin obtainable with the following method:

a) providing a CHO cell, which is
  adapted to growth in suspension culture,
  adapted to growth in serum-free medium, and
  mycoplasma free,

b) providing a nucleic acid comprising
  a prokaryotic origin of replication,
  a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,
  a second nucleic acid sequence encoding the heavy chain of said heterologous immunoglobulin, and a third nucleic acid sequence encoding the light chain of said heterologous immunoglobulin,

whereby a first transfection vector is provided which comprises said provided nucleic acid and an additional fourth nucleic acid sequence conferring resistance to a first eukaryotic selection agent,

whereby a second transfection vector is provided which comprises said provided nucleic acid and an additional fourth nucleic acid sequence conferring resistance to a second eukaryotic selection agent, whereby said second eukaryotic selection agent is different to said first eukaryotic selection agent,

b1) providing a nucleic acid comprising
  a prokaryotic origin of replication,
  a first nucleic acid sequence conferring resistance to a prokaryotic selection agent,
  a second nucleic acid sequence encoding the heavy chain of said heterologous immunoglobulin, and/or a third nucleic acid sequence encoding the light chain of said heterologous immunoglobulin,

whereby a third transfection vector is provided which comprises said provided nucleic acid and an additional fourth nucleic acid sequence conferring resistance to a third eukaryotic selection agent, whereby said third eukaryotic selection agent is different to said first eukaryotic selection agent and is also different to said second eukaryotic selection agent,

c) transfecting said CHO cell, wherein said transfecting comprises the following steps in the following order:
  (i) transfecting said CHO cell with said first transfection vector,
  (ii) selecting a CHO cell transfected in (i) by selected growth in cultivation medium containing a first eukaryotic selection agent to which the first transfection vector confers resistance,
  (iii) transfecting said selected CHO cell in (ii) with said second transfection vector,
  (iv) selecting a CHO cell transfected in (iii) by selected growth in cultivation medium containing said first eukaryotic selection agent to which the first transfection vector confers resistance and said second eukaryotic selection agent to which the second transfection vector confers resistance,
  (v) transfecting said CHO cell selected in (iv) with said third transfections vector,

(vi) selecting a CHO cell transfected in (v) by selected growth in a cultivation medium containing said first eukaryotic selection agent to which the first transfection vector confers resistance and said second eukaryotic selection agent to which the second transfection vector confers resistance and said third eukaryotic selection agent to which the third transfection vector confers resistance,

d) cultivating said transfected CHO cell in a medium in the presence of said first and said second eukaryotic selection agent, under conditions suitable for the expression of said second, and/or third nucleic acid, wherein said transfected CHO cell secretes the heterologous immunoglobulin and

e) recovering said secreted heterologous immunoglobulin from the cultivation medium and thereby producing a heterologous immunoglobulin in a CHO cell which is secreted to the cultivation medium,

wherein said resultant CHO cell is stable in the absence of any or all selection agents, as used in the previous steps, for up to generation 60.

* * * * *

EXHIBIT GG

(12) **United States Patent**
Zhou et al.

(10) **Patent No.: US 9,487,809 B2**
(45) **Date of Patent: Nov. 8, 2016**

(54) **DECREASING LACTATE LEVEL AND INCREASING POLYPEPTIDE PRODUCTION BY DOWNREGULATING THE EXPRESSION OF LACTATE DEHYDROGENASE AND PYRUVATE DEHYDROGENASE KINASE**

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CPC ***C12P 21/00*** (2013.01); ***C12N 1/38*** (2013.01); ***C12N 15/111*** (2013.01); ***C12N 15/113*** (2013.01); ***C12N 15/85*** (2013.01); *C12N 2310/14* (2013.01); *C12N 2320/00* (2013.01)

(58) **Field of Classification Search**
USPC 435/6.1, 69.1, 91.1, 91.31, 455, 3, 435/320.1, 325, 326; 536/23.1, 24.5
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

RE30,985	E	6/1982	Cartaya
4,515,893	A	5/1985	Kung et al.
4,560,655	A	12/1985	Baker
4,657,866	A	4/1987	Kumar
4,676,980	A	6/1987	Segal et al.
4,767,704	A	8/1988	Cleveland et al.
4,816,567	A	3/1989	Cabilly et al.
4,927,762	A	5/1990	Darfler
5,091,313	A	2/1992	Chang
5,122,469	A	6/1992	Mather et al.
5,622,700	A	4/1997	Jardieu et al.
5,672,347	A	9/1997	Aggarwal et al.
5,693,762	A	12/1997	Queen et al.
5,714,388	A	2/1998	Kusnetz
5,721,108	A	2/1998	Robinson et al.
5,726,856	A	3/1998	King, Jr. et al.
5,736,137	A	4/1998	Anderson et al.
2009/0209618	A1	8/2009	Dang et al.
2009/0215860	A1	8/2009	Morris et al.
2009/0325287	A1	12/2009	Dorai et al.

FOREIGN PATENT DOCUMENTS

EP	0 183 070	A2	6/1986
EP	0 183 070	A3	6/1986
EP	0 183 070	B1	6/1986
EP	0 244 234	A1	11/1987
EP	0 244 234	A3	11/1987
EP	0 244 234	B1	11/1987
EP	0 308 936	A2	3/1989
EP	0 308 936	A3	3/1989
EP	0 308 936	B1	3/1989
EP	0 402 226	A1	12/1990
EP	0 420 937	B1	11/1994
WO	WO-87/00195	A1	1/1987
WO	WO-90/03430	A1	4/1990
WO	WO-91/00360	A1	1/1991
WO	WO-92/20373	A1	11/1992
WO	WO-93/04173	A1	3/1993
WO	WO-93/08829	A1	5/1993
WO	WO-93/16185	A1	8/1993
WO	WO-95/19181	A1	7/1995

(Continued)

OTHER PUBLICATIONS

Chen et al (Biotech. & Bioeng., vol. 72, No. 1, pp. 55-61 (2001)).*
Jeong et al (Biochem. & Biophys. Res. Comm., vol. 289, pp. 1141-1149 (2001)).*
Kim et al Appl. Microbiol. Biotechnol., vol. 74, pp. 152-159 (2007)).*
Wigfield et al (Brit. J. Cancer, vol. 98, pp. 1975-1984 (2008)).*
Harris et al (Advan. Enzyme Regul., vol. 42, pp. 249-259 (2002)).*
Aruffo et al., "CD44 is the principal cell surface receptor for hyaluronate," *Cell* 61:1303-1313, (Jun. 29, 1990).

(Continued)

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(74) *Attorney, Agent, or Firm* — Morrison & Foerster LLP

(57) **ABSTRACT**

The present invention provides methods and compositions for reducing lactate production and increasing polypeptide production in cultured cells. In one aspect, the invention provides a method comprising culturing cells expressing a) a small interfering RNA (siRNA) specific for a lactate dehydrogenase (LDH) and b) an siRNA specific for a pyruvate dehydrogenase kinase (PDHK). In another aspect, the invention provides cultured cells or vectors comprising an siRNA specific for a LDH and an siRNA specific for a PDHK.

12 Claims, 12 Drawing Sheets

(56) **References Cited**

FOREIGN PATENT DOCUMENTS

WO	WO-95/23865	A1	9/1995
WO	WO-96/27011	A1	9/1996
WO	WO-96/30046	A1	10/1996
WO	WO-96/40210	A1	12/1996
WO	WO-97/26912	A2	7/1997
WO	WO-97/26912	A3	7/1997
WO	WO-97/26912	C1	7/1997
WO	WO-98/06248	A2	2/1998
WO	WO-98/06248	A3	2/1998
WO	WO-98/23761	A1	6/1998
WO	WO-98/45331	A2	10/1998
WO	WO-98/45331	A3	10/1998
WO	WO-98/51793	A1	11/1998
WO	WO-00/78348	A1	12/1998
WO	WO-01/00245	A2	1/2001
WO	WO-01/00245	A3	1/2001
WO	WO-01/40309	A2	6/2001
WO	WO-01/40309	A3	6/2001
WO	WO-02/04598	A2	1/2002
WO	WO-02/04598	A3	1/2002
WO	WO-2009/152413	A1	12/2009
WO	WO-2010/031074	A2	3/2010
WO	WO-2010/031074	A3	3/2010

OTHER PUBLICATIONS

Barnes et al., "Methods for growth of cultured cells in serum-free medium," *Anal. Biochem.* 102:255-270, (1980).

Baumgart et al., "L-lactate dehydrogenase A_4- and A_3B isoforms are bona fide peroxisomal enzymes in rat liver. Evidence for involvement in intraperoxisomal NADH reoxidation," *J. Biol. Chem.* 27(7):3846-3855, (Feb. 1997).

Berg et al. "Bispecific antibodies that mediate killing of cells infected with human immunodeficiency virus of any strain," *Proc. Natl. Acad. Sci. USA* 88:4723-4727 (Jun. 1991).

Bowton-Kinley et al., "Evidence for existence of tissue-specific regulation of the mammalian pyruvate dehydrogenase complex," *Biochem. J.* 329(Pt. 1):191-196, (Jan. 1998).

Brennan et al. "Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G_1 fragments," *Science* 229:81-83, (Jul. 5, 1985).

Brodeur Chapter 4 in Inc., New York, et al., "Mouse-human myeloma partners for the production of heterohybridomas," Chapter 4 in *Monoclonal Antibody Production Techniques and Applications,* Marcel Dekker, Inc., New York, New York, pp. 51-63, (1987).

Brüggermann et al., "Designer mice: The production of human antibody repertoires in transgenic animals," *Year in Immuno.* 7:33-40, (1993).

Carter et al., High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment, *Bio/Technology* 10:163-167 (Feb. 1992).

Carter et al. "Humanization of an antip185^{HER2} antibody for human cancer therapy," *Proc. Natl. Acad. Sci. USA* 89:4285-4289 (May 1992).

Ceriani et al. "Biological activity of two humanized antibodies against two different breast cancer antigens and comparison to their original murine forms," *Cancer Res.* 55(23):5852s-5856s (Dec. 1, 1995).

Chamow et al. "A humanized, bispecific immunoadhesin-antibody that retargets $CD3^+$ effectors to kill HIV-1-infected cells," *J. Immunol.* 153:4268-4280, (1994).

Chothia et al., "Canonical structures for the hypervariable regions of immunoglobulins," *J. Mol. Biol.* 196:901-917, (1987).

Choy et al., "Percentage of anti-cd4 monoclonal antibody-coated lymphocytes in the rheumatoid joint is associated with clinical improvements," *Arthritis Rheum.* 39(1):52-56, (Jan. 1996).

Clackson et al., "Making antibody fragments using phage display libraries," *Nature* 352:624-628, (Aug. 15, 1991).

Dhainaut et al., "CDP571, a humanized antibody to human tumor necrosis factor-α: Safety, pharmacokinetics, immune response, and influence of the antibody on cytokine concentrations in patients with septic shock," *Crit. Care Med.* 23(9):1461-1469, (Sep. 1995).

Dietl et al., "Lactic acid and acidification inhibit TNF secretion and glycolysis of human monocytes," *J. Immunol.* 184(3):1200-1209, (Feb. 1, 2010).

Duchosal et al., "Immunization of hu-PBL-SCID mice and the rescue of human monoclonal Fab fragments through combinatorial libraries," *Nature* 355:258-262, (Jan. 16, 1992).

Ellis et al., "Engineered anti-cd38 monoclonal antibodies for immunotherapy of multiple myeloma," *J. Immunol.* 155(2):925-937, (1995).

Goding., "Production of monoclonal antibodies," Chapter 3 in *Monoclonal Antibodies: Principles and Practice,* Academic Press, London, England, pp. 59-103. (1983).

Graham et al. "Characteristics of a human cell line transformed by DNA from human adenovirus type 5," *J. Gen Virol.* 36:59-72, (1977).

Graziano et al., "Construction and characterization of a humanized anti-γ-lg receptor type I (FcγRl) monoclonal antibody," *J. Immunol.* 155(10):4996-5002, (1995).

Gruber et al., "Efficient tumor cell lysis mediated by a bispecific single chain antibody expressed in *Escherichia coli,*" *J. Immunol.* 152:5368-5374, (1994).

Ham et al., "Media and growth requirements," *Meth. Enz.* 58:44-93, (1979).

Holliger et al. "Diabodies: Small bivalent and bispecific antibody fragments," *Proc. Natl. Acad. Sci. USA* 90:6444-6448, (Jul. 1993).

Hoogenboom et al., "By-passing Immunisation. Human antibodies from synthetic repertoires of germline V_H gene segments rearranged in Vitro," *J. Mol. Biol.* 227:381-388, (1991).

Hoogenboom et al., "Construction and expression of antibody-tumor necrosis factor fusion proteins," *Mol. Immunol.* 28(9):1027-1037, (1991).

Hourmant et al., Administration of an anti-CD11a monoclonal antibody in recipients of kidney transplantation, *Transplantation* 58:377-380 ,(Aug. 1994).

International Search Report mailed on Jan. 18, 2012, for PCT Application No. PCT/US11/38191, filed on May 26, 2011, one page.

Jakobovits et al., "Analysis of homozygous mutant chimeric mice: Deletion of the immunoglobulin heavy-chain joining region blocks B-cell development and antibody production," *Proc. Natl. Acad. Sci. USA* 90:2551-2555, (Mar. 1993).

Jakobovitis et al., "Germ-line transmission and expression of a human-derived yeast artificial chromosome," *Nature* 362:255-258, (Mar. 18, 1993).

Jones et al., "Replacing the complementarity-determining regions in a human antibody with those from a mouse," *Nature* 321:522-525, (May 29, 1986).

Jurcic et al., "Radiolabled anti-CD33 monoclonal antibody M195 for myeloid leukemias," *Cancer Res.* 55(23 Suppl.):5908s-5910s, (Dec. 1, 1995).

Juweid et al., "Treatment of non-hodgkin's lymphoma with radiolabeled murine, chimeric, or humanized LL2, an anti-CD22 monoclonal antibody," *Cancer Res.* 55(23 Suppl.):5899s-5907s, (Dec. 1, 1995).

Kim et al., "The vascular endothelial growth factor proteins: Growth factor proteins: Identification of biologically relevant regions by neutralizing monoclonal antibodies," *Growth Factors* 7:53-64, (1992).

Kim et al., "Down-regulation of lactate dehydrogenase-A by siRNAs for reduced lactic acid formation of Chinese hamster ovary cells producing thrombopoietin," *Appl. Microbiol. Biotechnol.* 74(1):152-159, (Nov. 4, 2006).

Köhler et al., "Continuous cultures of fused cells secreting antibody of predefined specificity," *Nature* 256:495-497, (1975).

Kostelny et al., "Formation of a bispecific antibody by the use of leucine zippers," *J. Immunol.* 148(5):1547-1553, (Mar. 1, 1992).

Kozbor et al. "A human hybrid myeloma for production of human monoclonal antibodies," *J. Immunol.* 133(6):3001-3005, (Dec. 1984).

(56) **References Cited**

OTHER PUBLICATIONS

Kuystermans et al. "Using cell engineering and omic tools for the improvement of cell culture processes," *Cytotechnology* 53(1-3):3-22, (Apr. 2007, e-pub. Feb. 24, 2007).
Li et al., "Evolutionary relationships of vertebrate lactate dehydrogenase isozymes A_4 (muscle), B_4 (heart), and C_4 (testis)," *J. Biol. Chem.* 258(11):7029-7032, (Jun. 10, 1983).
Lindmark et al., "Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera," *J. Immunol. Meth.* 62:1-13, (1983).
Litton et al., "Antibody-targeted superantigen therapy induces tumor-infiltrating lymphocytes, excessive cytokine production, and apoptosis in human colon carcinoma," *Eur. J. Immunol.* 26(1):1-9, (1996).
Liu et al., "Lactate inhibits lipolysis in fat cells through activation of an orphan G-protein-coupled receptor, GPR81," *J. Biol. Chem.* 284(5):2811-2822, (Jan. 30, 2009).
Lorenz et al., "In vivo blockade of TNF-α by Intravenous infusion of a chimeric monoclonal TNF-α antibody in patients with rheumatoid arthritis," *J. Immunol.* 156(4):1646-1653, (1996).
Lu et al., "Induction of Pyruvate Dehydrogenase Kinase-3 by Hypoxia-inducible Factor-1 Promotes Metabolic Switch and Drug Resistance," *J. Biol. Chem.* 283(42):28106-28114, (Oct. 17, 2008).
Marks et al., "By-passing Immunization. Human antibodies from V-gene libraries displayed on phage," *J. Mol. Biol.* 222:581-597, (1991).
Marks et al., "By-passing immunization: Building high affinity human antibodies by chain shuffling," *Bio/Technology* 10:779-783 (Jul. 1992).
Mather., "Establishment and characterization of two distinct mouse testicular epithelial cell lines," *Biol. Reproof.* 23:243-251, (1980).
Mather et al., "Culture of testicular cells in hormone-supplemented serum-free medium," *Annals N.Y. Acad. Sci.* 383:44-68, (1982).
Mathupala et al., "Hexokinase-2 bound to mitochondria: Cancer's stygian link to the 'Warburg effect' and a pivotal target for effective therapy," *Seminars in Cancer Biology* 19:17-24, 17 pages, (Feb. 2009).
McCafferty et al., "Phage antibodies: filamentous phage displaying antibody variable domains," *Nature* 348:552-554, (Dec. 6, 1990).
Milstein et al., "Hybrid hybridomas and their use in immunohistochemistry," *Nature* 305:537-540, (Oct. 6, 1983).
Morimoto et al., "Single-step purification of $F(ab')_2$ fragments of mouse monoclonal antibodies (immunoglobulins G1) by hydrophobic interaction high performance liquid chromatography using TSKgel Phenyl-5PW," *Journal of Biochemical and Biophysical Methods* 24:107-117, (1992).
Morrison et al., "Chimeric human antibody molecules: Mouse antigen binding domains with human constant region domains," *Proc. Natl. Acad. Sci.* USA 81:6851-6855, (Nov. 1984).
O'Callaghnan et al., Systems biotechnology of mammalian cell factories, *Brief Funct. Genomic Proteomic* 7(2):95-110, (Mar. 2008).
Patel et al., "Regulation of mammalian pyruvate dehydrogenase complex by phosphorylation: complexity of multiple phosphorylation sites and kinases," *Exp. Mol. Med.* 33(4):191-197, (Dec. 2001).
Presta., "Antibody engineering," *Curr. Op. Struct. Biol.* 2:593-596, (1992).
Presta et al. "Humanization of an antibody directed against IgE," *J. Immunol.* 151(5):2623-2632, (Sep. 1, 1993).
Richman et al., "Radioimmunotherapy for breast cancer using escalating fractionated doses of ^{131}l-labeled chimeric L6 antibody with peripheral blood progenitor Cell," *Cancer Res.* 55(23 Suppl.): 5916s-5920s, (Dec. 1, 1995).
Riechmann et al., "Reshaping human antibodies for therapy," *Nature* 332:323-329, (Mar. 24, 1988).
Rutz et al., "Towards in vivo application of RNA interference—new toys, old problems," *Arthritis Research and Therapy* 6(2):78-85 (2004, e-pub. Mar. 10, 2004).
Samuvel et al., "Lactate boosts TLR4 signaling and NF-κB pathway-mediated gene transcription in macrophages via monocarboxylate transporters and MD-2 up-regulation," *J. Immunol.* 182(4):2476-2484, (Feb. 2009).
Shalaby et al., "Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the *HER2* protooncogene," *J. Exp. Med.* 175:217-225, (Jan. 1, 1992).
Sharkey et al., Evaluation of a complementarity-determining region-grafted (humanized) anti-carcinoembryonic antigen monoclonal antibodies, *Cancer Res.* 55(23 Suppl.):5935s-5945s, (Dec. 1, 1995).
Sims et al., "A humanized cd18 antibody can block function without cell destruction," *J. Immunol.* 151(4):2296-2308, (Aug. 15, 1993).
Skory., "Isolation and expression of lactate dehydrogenase genes from *Rhizopus oryzae,*" *Appl. Envion. Microbiol.* 66(6):2343-2348, (Jun. 2000).
St. John et al., "Immunologic therapy for ARDS, septic shock, and multiple-organ failure," *Chest* 103:932-943, (1993).
Stamenkovic et al., "The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45RO on t cells and α2-6 Sialyltransferase, CD75, on B cells," *Cell* 66:1133-1144, (Sep. 20, 1991).
Traunecker et al., "Bispecific single chain molecules (Janusins) target cytotoxic lymphocytes on HIV infected cells," *EMBO J.* 10(12):3655-3659, (1991).
Tutt et al., "Trispecific F(ab')3 derivatives that use cooperative signaling via the tcr/cd3 complex and cd2 to activate and redirect resting cytotoxic t cells," *J. Immunol.* 147(1):60-69, (Jul. 1, 1991).
Urlaub et al., "Isolation of Chinese hamster cell mutations deficient in dihydrofolate reductase activity," *Proc. Natl. Acad. Sci. USA* 77(7):4216-4220, (Jul. 1980).
Vaughan et al., "Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library," *Nature Biotech.* 14:309-314, (Mar. 1996).
Verhoeyen et al., "Reshaping human antibodies: Grafting an antilysozyme activity," *Science* 239:1534-1536, (1988).
Waterhouse et al., Combinatorial infection and in vivo recombination: a strategy for making large phage antibody repertoires, *Nuc. Acids Res.* 21(9):2265-2266, (1993).
Wigfield et al., "PDK-1 regulates lactate production in hypoxia and is associated with poor prognosis in head and neck squamous cancer," *British Journal of Cancer* 98:1975-1984, (Jun. 10, 2008).
Written Opinion mailed on Jan. 18, 2012, for PCT Application No. PCT/US11/38191, filed on May 26, 2011, ten pages.
Zapata et al., "Engineering linear $F9ab')_2$ fragments for efficient production in *Escherichia coli* and enhanced antiproliferative activity," *Protein Eng.* 8(10):1057-1062, (1995).
Zhou et al., "Decreasing lactate level and increasing antibody production in Chinese hamster ovary cells (CHO) by reducing the expression of lactate dehydrogenase and pyruvate dehydrogenase kinases," *Journal of Biotechnology* 153:27-34, (2011, e-pub. Mar. 8, 2011).
Chen et al., "Engineering of a mammalian cell line for reduction of lactate formation and high monoclonal antibody production," *Biotechnol. Bioeng.* 72(1):55-61, (Jan. 5, 2001).
Cruz et al., "Effects of ammonia and lactate on growth, metabolism, and productivity of BHK cells," *Enzyme Microb. Technol.* 27(1-2):43-52, (Jul. 1, 2000).
Elbashier et al., "Analysis of gene function in somatic mammalian cells using small interfering RNAs," *Methods* 26:199-213, (2002).
Glacken et al., "Mathematical descriptions of hybridoma culture kinetics: I. Initial metabolic rates," *Biotechol. Bioeng.* 66(3):171-179, (1988).
Harris et al., (2002). "Regulation of the activity of the pyruvate dehydrogenase complex," *Adv. Enzyme Regul.* 42:249-259, (2002).
Holness et al. "Regulation of pyruvate dehydrogenase complex activity by reversible phosphorylation," *Biochem Soc. Trans.* 31(Pt. 6):1143-51, (Dec. 2003).
Irani et al., "Improvement of the primary metabolism of cell cultures by introducing a new cytoplasmic pyruvate carboxylase reaction," *Biotechnol. Bioeng.* 66(4):238-246, (1999).

(56) **References Cited**

OTHER PUBLICATIONS

Jeong et al., "Blocking of acidosis-mediated apoptosis by a reduction of lactate dehydrogenase activity through antisense mRNA expression," *Biochem. Biophys Res. Commun.* 289(5):1141-1149, (Dec. 21, 2001).
Langheinrich et al., "Control of pH in large-scale, free suspension animal cell bioreactors: alkali addition and pH excursions," *Biotechnol. Bioeng.* 66(3):171-179, (1999).
Lao et al., "Effects of ammonium and lactate on growth and metabolism of a recombinant Chinese hamster ovary cell culture," *Biotechnol Prog.* 13(5):688-691, (Sep.-Oct. 1997).
Rao et al., "siRNA vs. shRNA: Similarities and Differences," *Advanced Drug Delivery Reviews* 61:746-759, (2009).
Read et al., "Structural basis for altered activity of M- and H-isozyme forms of human lactate dehydrogenase," *Proteins* 43(2)175-185, (May 2001).
Roche et al., "Pyruvate dehydrogenase kinase regulatory mechanisms and inhibition in treating diabetes, heart ischemia, and cancer," *Cell Mol. Life Sci.* 64(7-8):830-849, (Apr. 2007).
Stoppa et al., "Anti-LFA1 monoclonal antibody (25.3) for treatment of steroid-resistant grade III-IV acute graft-versus-host disease," *Transplant Intl.* 4:3-7, (1991).
Fahrner et al., "Performance comparison of protein A affinity-chromatography sorbents for purifying recombinant monoclonal antibodies," *Biotechnol. Appl. Biochem.* 30(Part 2):121-128, (Oct. 1999).
Davis et al., "Lymphoid Cell Functions During Copper Deficiency," *Nutrition Research* 7:211-222, (1987).
Gu et al., "Lactate and Pyruvate Levels in Blood and Cerebrospinal Fluid in Patients with Menkes Disease," *The Journal of Pediatrics* 164:890-894, (2014).
Luo et al., "Comparative Metabolite Analysis to Understand Lactate Metabolism Shift in Chinese Hamster Ovary Cell Culture Process," *Biotechnology and Bioengineering* 109(1):146-156, (Jan. 2012).
Patel et al., "Regulation of the Pyruvae Dehydrogenase Complex," *Biochemical Society Transactions* 34(2):217-222, (2006).
Wu et al. "RNA Interference Technology to 1-46 Improve Recombinant Protein Production in Chinese hamster ovary cells," *Biotechnology Advances* 27(4):417-422, (Jul. 1, 2009).
Zagari et al., "Lactate Metabolism Shift in CHO Cell Culture: The Role of Mitochondrial Oxidative Activity," *New Biotechnology* 30(2):238-245, (Jan. 2013).
Supplemental European Search Report mailed on Jun. 3, 2014, for European Patent Application No. 11787435.4, filed on May 26, 2011, 7 pages.

* cited by examiner

Case 1:18-cv-00095-CFC Document 1-3 Filed 01/12/18 Page 442 of 681 PageID #: 1311

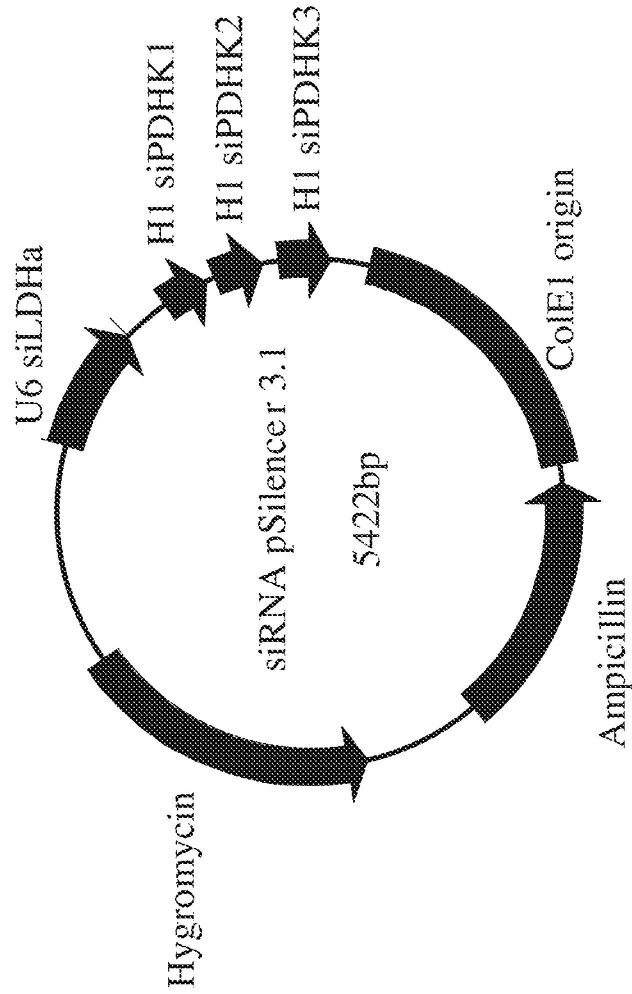

Figure 1

Figure 2

Relative mRNA Expression

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

LDHa
PDHK1
PDHK2
PDHK3

siRNA#1
siRNA#2
siRNA#3
siRNA#4
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siRNA#6
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siRNA#8
siRNA#9
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siRNA#11
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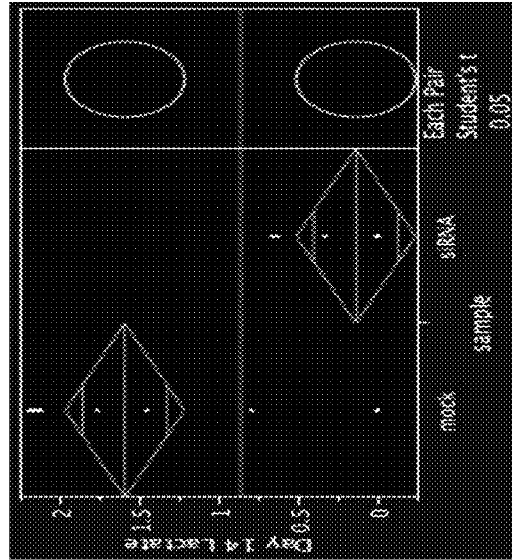
mock



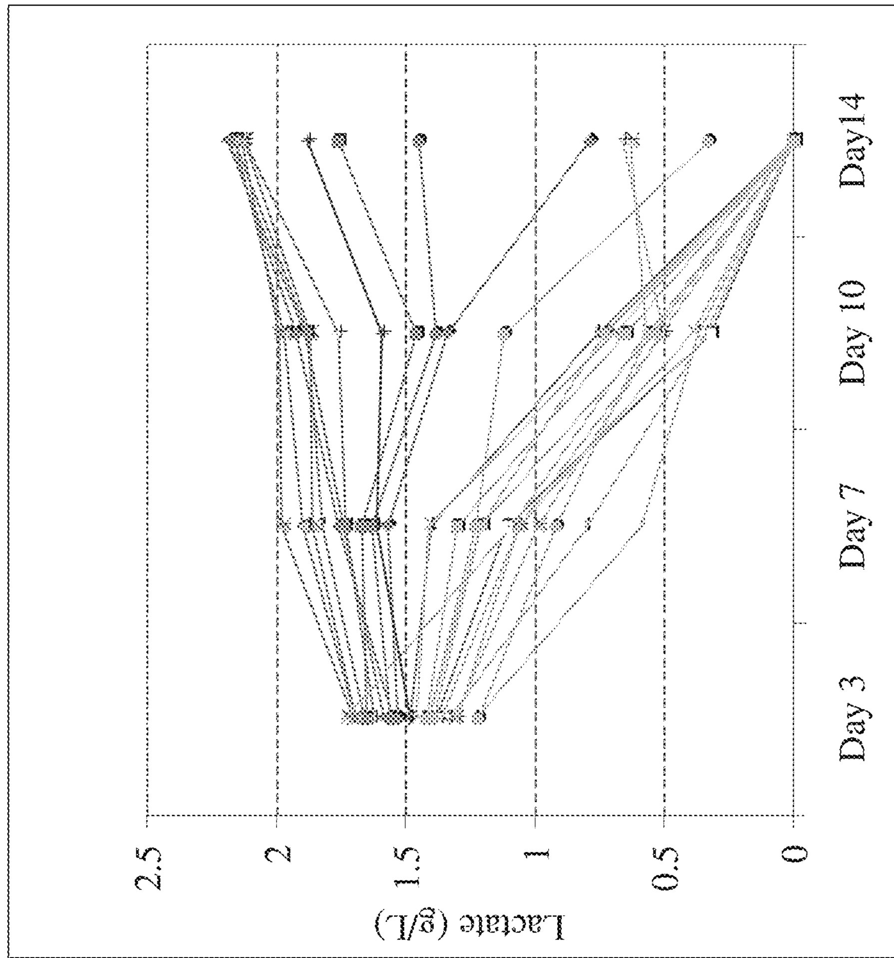


Figure 3A

Figure 3B

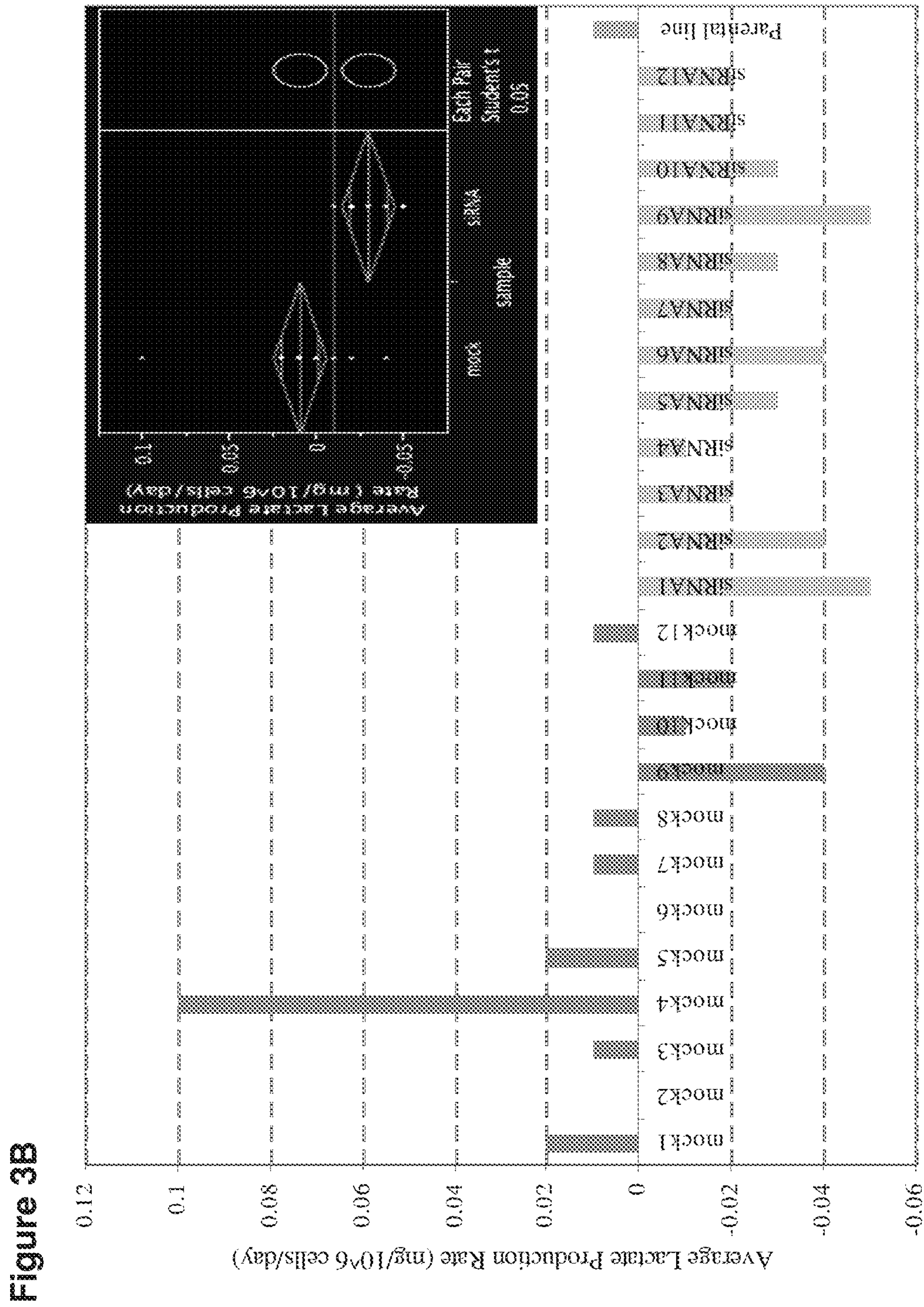
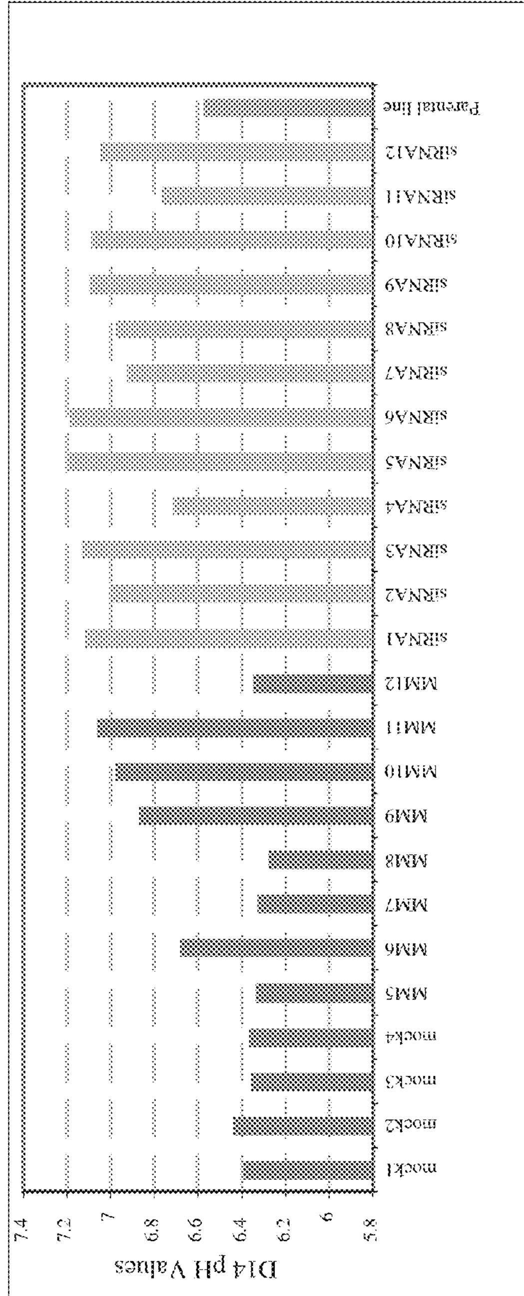


Figure 3C



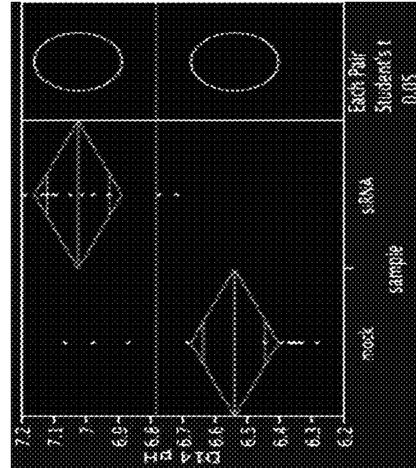
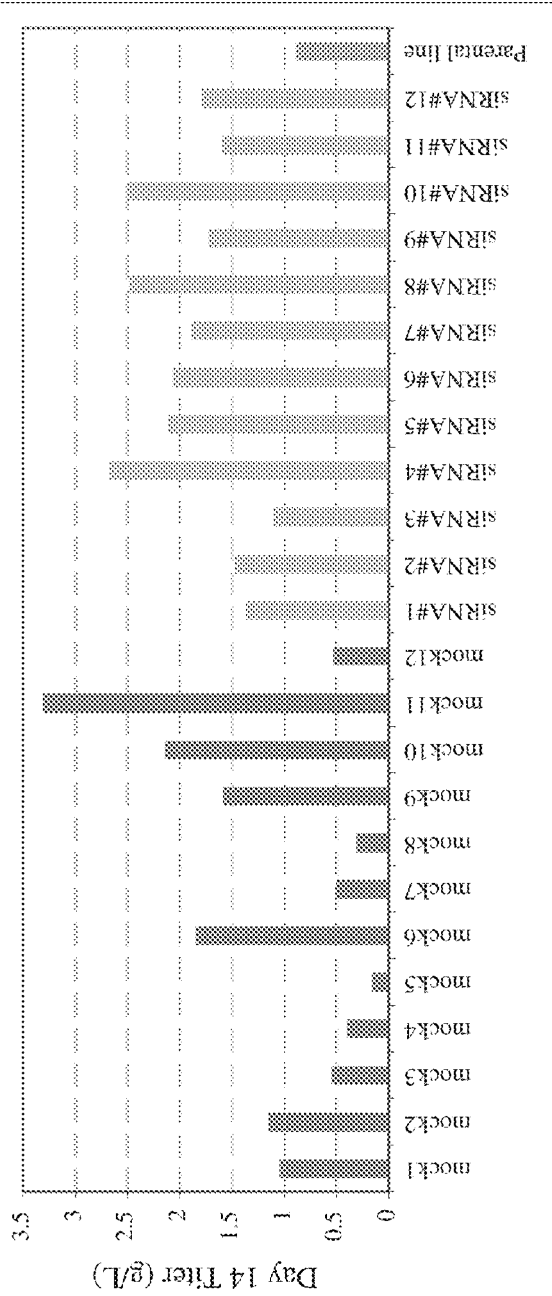


Figure 4A



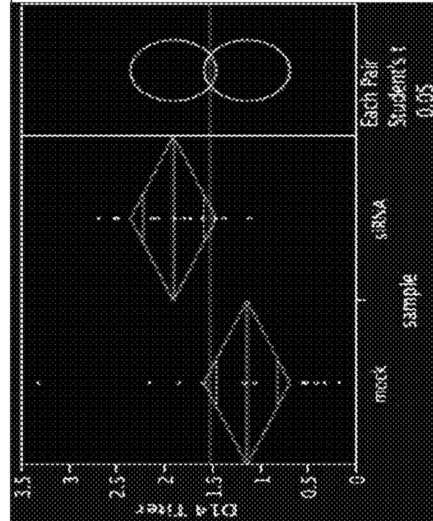
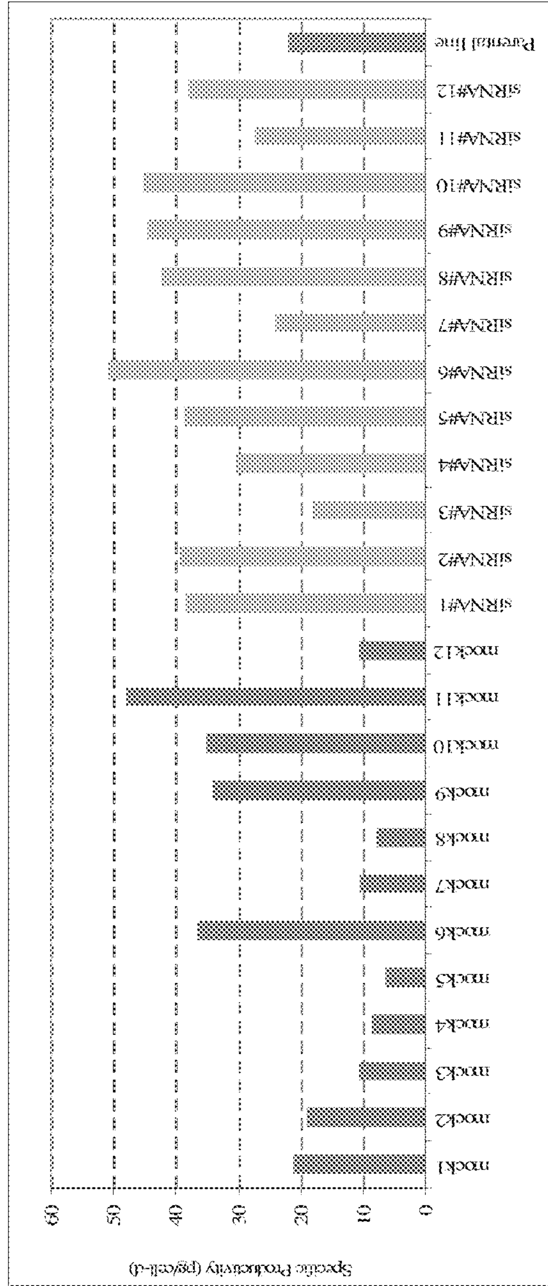
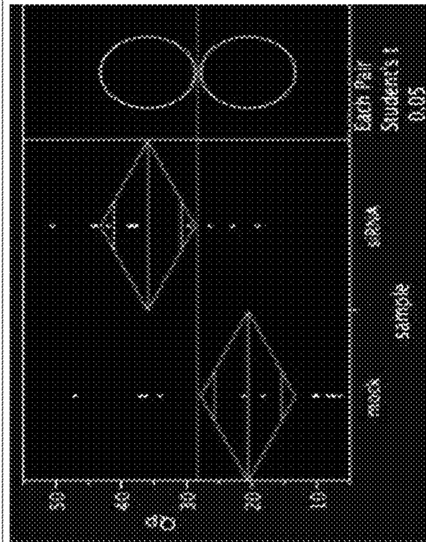
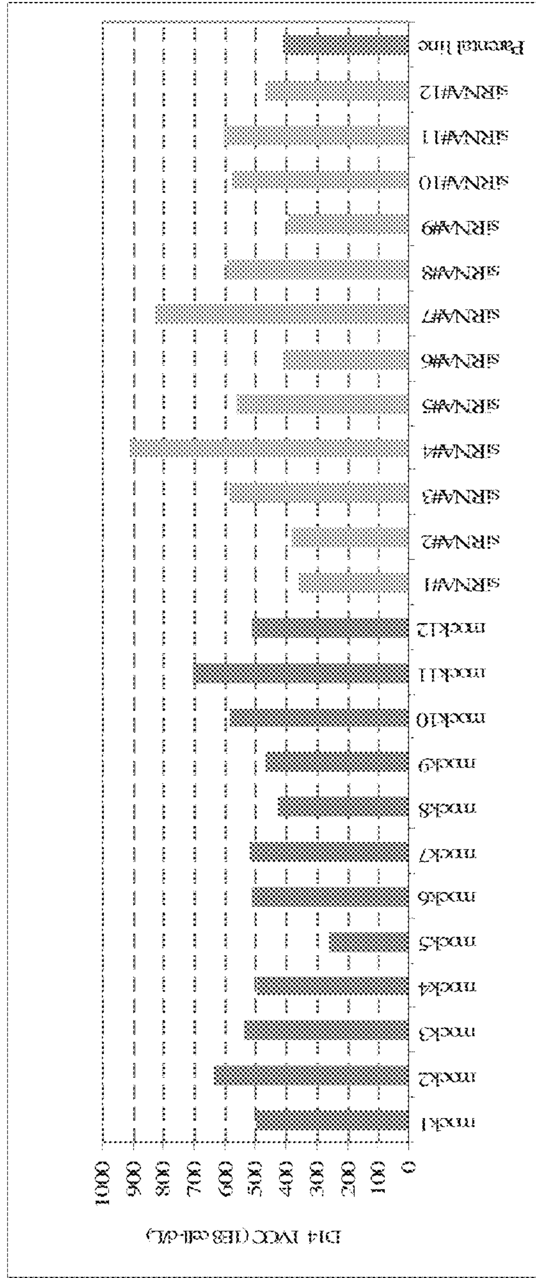


Figure 4B







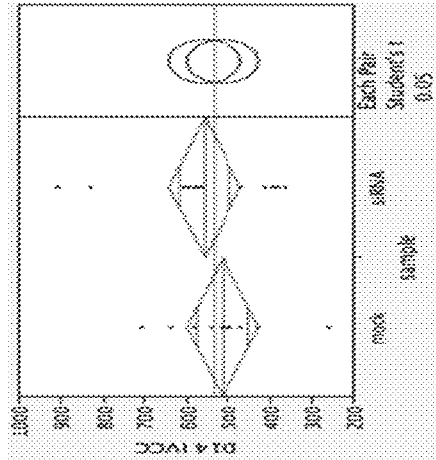


Figure 4C

Figure 5A

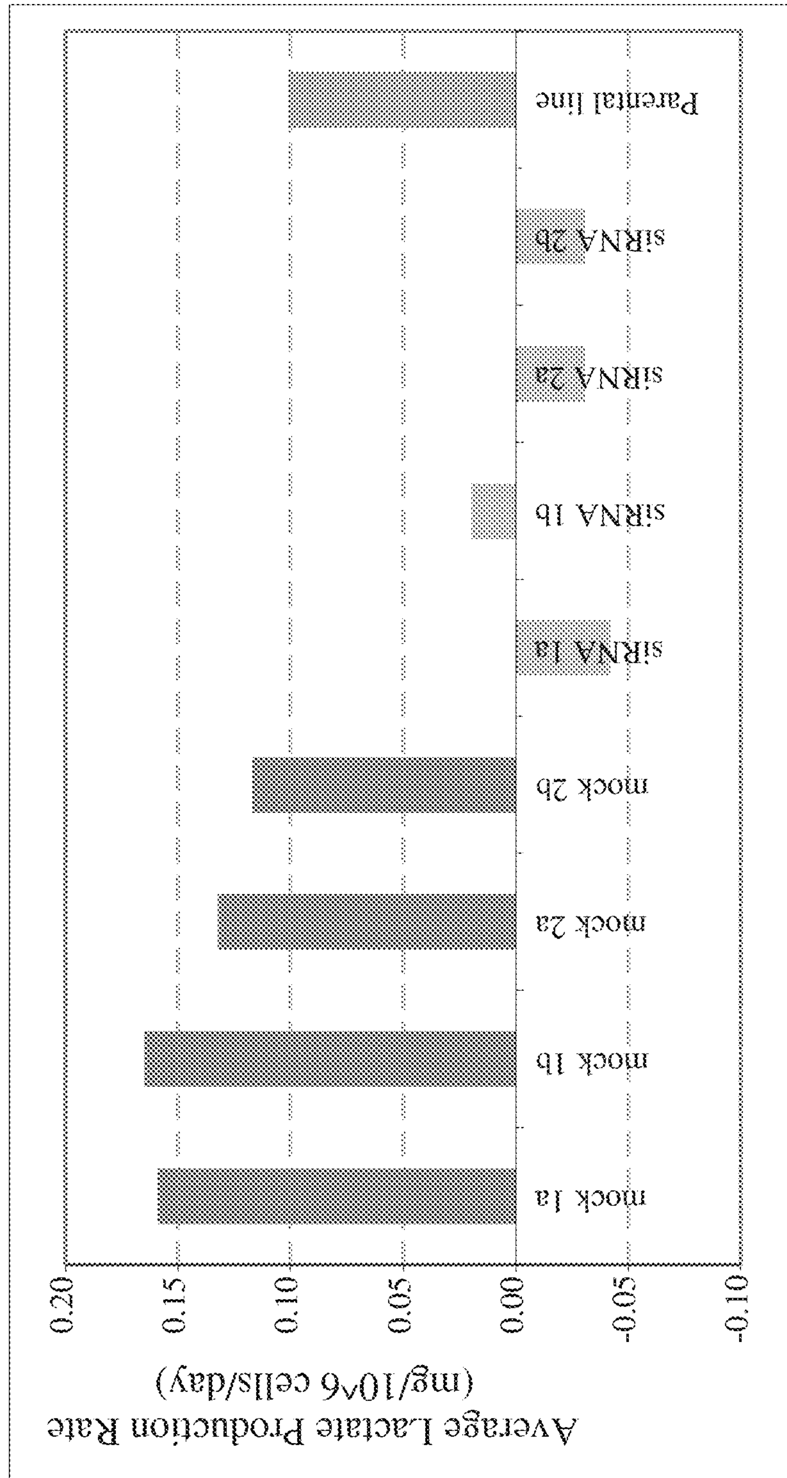


Figure 5B

Figure 5C

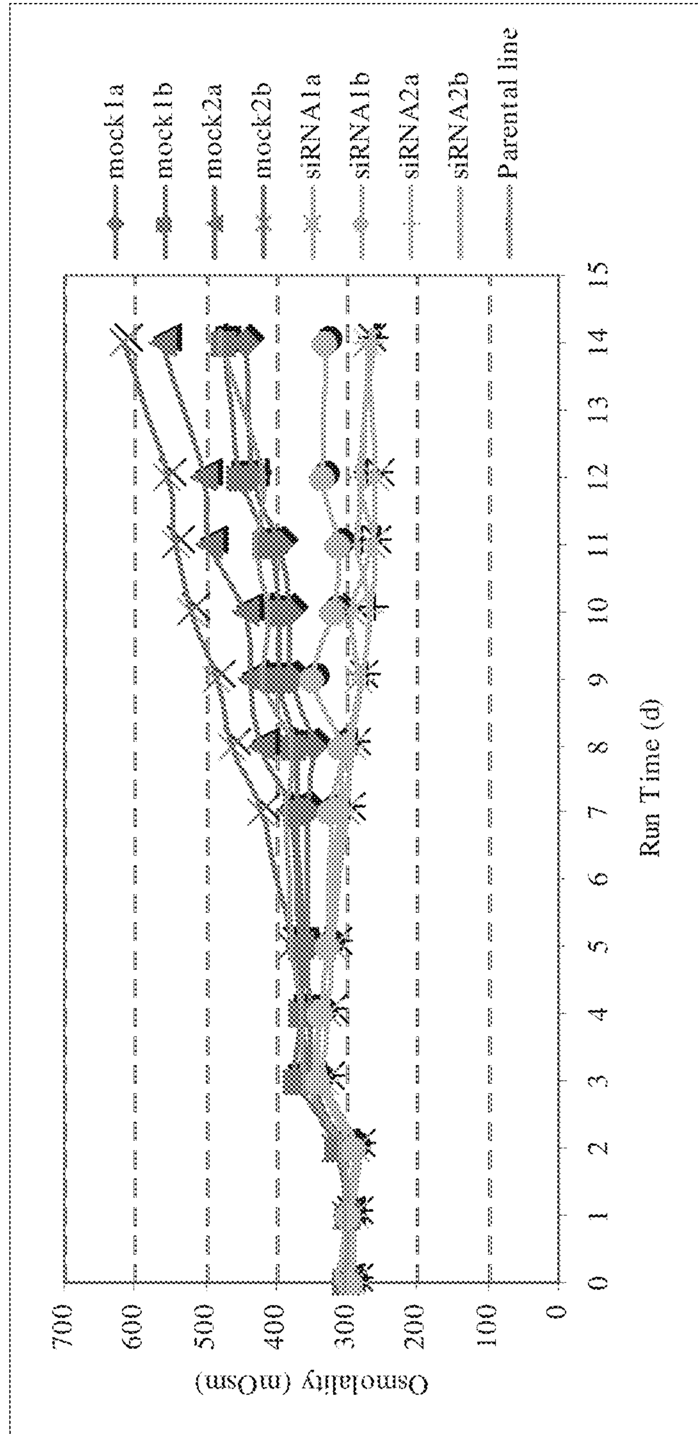


Figure 6

Titer (mg/L)

3500
3000
2500
2000
1500
1000
500
0

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Run Time (d)

mock 1a
mock 2a
siRNA 1a
siRNA 1b
siRNA 2a
siRNA 2b
mock 1b
mock 2b
Parental line

DECREASING LACTATE LEVEL AND INCREASING POLYPEPTIDE PRODUCTION BY DOWNREGULATING THE EXPRESSION OF LACTATE DEHYDROGENASE AND PYRUVATE DEHYDROGENASE KINASE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of International Patent Application No. PCT/US2011/038191, filed May 26, 2011; which claims priority benefit to U.S. Provisional Patent Application No. 61/349,727 filed May 28, 2010, the content of each of which is hereby incorporated herein by reference in its entirety.

SEQUENCE LISTING

The instant application contains a Sequence Listing submitted via EFS-Web and hereby incorporated by reference in its entirety. Said ASCII copy, created on Nov. 15, 2012, is named 146392007800, and is 3.83 bytes in size.

FIELD OF THE INVENTION

The field of this invention relates generally to methods and compositions for reducing lactate production and increasing polypeptide production in cultured cells.

BACKGROUND OF THE INVENTION

Biopharmaceutical market is growing rapidly, and the industry is projected to reach $70 billion dollars by year 2010. See Genetic Engineering in Livestock: New Applications and Interdisciplinary Perspectives (Engelhard et al., 2009) Springer Berlin Heidelberg. Given the increase in demand in therapeutic proteins and the increase in competitions in market sharing among companies, there is a need in improving technologies to achieve better productivity in therapeutic proteins. Towards this goal, different approaches, such as host cell engineering, have been explored. See Kuystermans et al., *Cytotechnology* 53(1-3): 3-22 (2007); and O'Callaghan and James, *Brief Funct. Genomic Proteomic* 7(2):95-110 (2008). Cultured cells, such as Chinese Hamster Ovary (CHO) cells, are widely used to produce therapeutic proteins. For example, pH-controlled fed-batch bioreactor culture has been used widely to produce recombinant monoclonal antibodies. Langheinrich and Nienow, *Biotechnol. Bioeng.* 66(3):171-9 (1999). Lactate is one of the main accumulated waste products during fed-batch culture, and it has been shown to inhibit cell growth and protein production. See Glacken et al., *Biotechnol. Bioeng.* 32:491-506 (1988); and Lao and Toth, *Biotechnol. Prog.* 13:688-691 (1997). This in turn leads to an increase in the amount of alkali needed for adding into the culture medium to control the pH. Dietl et al., *J. Immunol.* 184(3):1200-9 (2010); Langheinrich and Nienow, *Biotechnol. Bioeng,* 66(3):171-9 (1999). Increased addition of alkali to the cell culture medium for maintaining the pH can result in an increase in osmolality, and this increase can lead to cell growth inhibition and decreased antibody productivity. Cruz et al., *Enzyme Microb. Technol.* 27(1-2):43-52 (2000); Iran et al., *Biotechnol. Bioeng.* 66:238-246 (1999). Hence, reducing the lactate level is desired for the development of polypeptide or a higher titer antibody production process.

There are many factors that can influence lactate production in cell culture, such as controlling the pyruvate level. See Liu et al., *J. Biol. Chem.,* 284(5):2811-22 (2009); and Samuvel et al., *J. of Immunol.* 182(4):2476-84 (2009). Pyruvate is the substrate for the enzymes pyruvate dehydrogenase (PDH) and lactate dehydrogenase (LDH).

The PDH complex is a multi-enzyme unit consisting of three catalytic enzymes, E1, E2, and E3. Patel and Korotchkina, *Exp. Mol. Med.* 33(4):191-7 (2001). This complex catalyzes the rate-limiting conversion reaction in converting from pyruvate to acetyl-CoA, which is the entry point of tricarboxylic acid (TCA) cycle. The activity of PDH is regulated by the enzymes pyruvate dehydrogenase kinases (PDHK(s)) and pyruvate dehydrogenase phosphatases (PDHPs). PDHKs phosphorylate PDH to suppress its enzymatic activity, whereas PDHP dephosphorylate and thus activate PDH. See Patel and Korotchkina, *Exp. Mol. Med.* 33(4):191-7 (2001); Roche and Hiromasa, *Cell Mol. Life. Sci.* 64(7-8):830-49 (2007); Holness and Sugden, *Biochemical Society Transactions,* 31:1143-1151 (2003). There are four isotypes of PDHK in mammalian cells (PDHK1, PDHK2, PDHK3, and PDHK4) with tissue specific distributions. See Harris et al., *Adv. Enzyme Regul.* 42:249-59 (2002); and Bowker-Kinley et al., *Biochem. J.* 329(1):191-6 (1998).

LDH directly catalyzes the interconversion of pyruvate and lactate with concurrent interconversion of NADH and NAD+. In mammalian cells, LDHs exist as either homo- or heterotetramers consisting mostly A and B subunits (or H and M subunits, respectively) encoded by LDHa and LDHb genes, and sometimes homotetramers of C subunit encoded by LDHc genes. See Baumgart et al., *J. Biol. Chem.* 271 (7):3846-55 (1996); Li et al., *J. Biol. Chem.* 258(11):7029-32 (1983); Skory C. D., *Appl. Environ. Microbiol.* 66(6): 2343-8 (2000); and Read et al., *Proteins* 43(2):175-185 (2001). For example, in CHO cells, LDH isotypes have been shown to be intermediates of the A3B and A2B2 tetramer. Jeong et al., *Biochem. Biophys. Res. Commun.* 289(5): 1141-9 (2001). Previous studies have shown that downregulating LDHa in CHO cells by disrupting the gene via homologous recombination (Chen et al., *Biotechnol. Bioeng.* 72(1):55-61 (2001)), antisense technology (Jeong et al., *Biochem. Biophys. Res. Commun.* 289(5):1141-9 (2001)), or small or short interfering RNA (siRNA) (Kim and Lee, *Appl. Microbiol. Biotechnol.* 74(1):152-9 (2007)) can reduce lactate level, but did not achieve appreciable improvement in protein productivity. For example, in the case of LDHa specific siRNA, even though there was reportedly a 45-79% reduction in lactate level, there was no significant improvement in Specific Productivity (Qp) and product (antibody) titer, suggesting that knocking down LDHa alone in CHO cells is not sufficient to improve Qp and product yield efficiently. Thus, more efficient methods for reducing lactate production are needed for achieving a better therapeutic polypeptide production.

All publications, patents, and patent applications cited herein are hereby incorporated by reference herein in their entirety for all purposes to the same extent as if each individual publication, patent, and patent application were specifically and individually indicated to be so incorporated by reference.

BRIEF SUMMARY OF THE INVENTION

The present invention provides methods and compositions for reducing lactate production and increasing polypeptide production in cultured cells. The inventors have discovered that concomitant downregulation of a LDH and PDHKs via siRNAs in cultured cells expressing polypep-

tides (e.g., antibodies) decreased lactate level, lactate production rate, and osmolality, and increased specific polypeptide productivity (e.g., Specific Productivity) and polypeptide production (e.g., productivity). Further, these cultured cells with downregulated LDH and PDHKs exhibited no negative impact on cell growth, cell viabilities, and the quality of polypeptides produced.

In one aspect, the invention provides a method for reducing lactate production in cultured cells, the method comprising culturing cells expressing a) a small interfering RNA (siRNA) specific for a lactate dehydrogenase (LDH) and b) an siRNA specific for a pyruvate dehydrogenase kinase (PDHK).

In another aspect, the invention provides cells in culture comprising a) an siRNA specific for a LDH and an siRNA specific for a PDHK.

In some embodiments, the cultured cells further express an siRNA specific for a second PDHK. In some embodiments, the cultured cells further express an siRNA specific for a third PDHK. In some embodiments, the cultured cells further express an siRNA specific for a fourth PDHK.

In another aspect, the invention provides a method for reducing lactate production in cultured cells, the method comprising culturing cells comprising a first heterologous nucleic acid sequence encoding a small interfering RNA (siRNA) specific for a lactate dehydrogenase (LDH) and a second heterologous nucleic acid sequence encoding an siRNA specific for a pyruvate dehydrogenase kinase (PDHK), wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter.

In another aspect, the invention provides cells in culture comprising a first heterologous nucleic acid sequence encoding a first siRNA specific for a LDH and a second heterologous nucleic acid sequence encoding a second siRNA specific for a PDHK, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter.

In some embodiments, the cells further comprise a third heterologous nucleic acid sequence encoding an siRNA specific for a second PDHK and wherein the third heterologous nucleic acid sequence is operably linked to a third promoter. In some embodiments, the cells further comprise a fourth heterologous nucleic acid sequence encoding an siRNA specific for a third PDHK and wherein the fourth heterologous nucleic acid sequence is operably linked to a fourth promoter. In some embodiments, the cells further comprise a fifth heterologous nucleic acid sequence encoding an siRNA specific for a fifth PDHK and wherein the fifth heterologous nucleic acid sequence is operably linked to a fifth promoter.

In some embodiments, the LDH is LDHa, LDHb, or LDHc.

In some embodiments, the PDHK is selected from the group consisting of PDHK1, PDHK2, PDHK3, and PDHK4. In some embodiments, the PDHK is selected from the group consisting of PDHK1, PDHK2, and PDHK3. In some embodiments, the PDHK is selected from the group consisting of PDHK1 and PDHK2. In some embodiments, the PDHK is selected from the group consisting of PDHK1 and PDHK3. In some embodiments, the PDHK is selected from the group consisting of PDHK2 and PDHK3.

In some embodiments, the method for reducing lactate production in cultured cells comprises culturing cells comprising a first heterologous nucleic acid sequence encoding an siRNA specific for a lactate dehydrogenase (LDH) and a second, third, and fourth heterologous nucleic acid sequences encoding three different siRNAs specific for a first, second, and third PDHKs, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second, third, and fourth heterologous nucleic acid sequences are operably linked to a second, third, and fourth promoters, respectively. In some embodiments, the LDH is LDHa, wherein the first PDHK is PDHK1, the second PDHK is PDHK2, and the third PDHK is PDHK3.

In some embodiments, the cells in culture comprises a first heterologous nucleic acid sequence encoding a first siRNA specific for a LDH and a second, third, and fourth heterologous nucleic acid sequences encoding three different siRNAs specific for a first, second, and third PDHKs, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second, third, and fourth heterologous nucleic acid sequences are operably linked to a second, third, and fourth promoters, respectively. In some embodiments, the LDH is LDHa, wherein the first PDHK is PDHK1, the second PDHK is PDHK2, and the third PDHK is PDHK3.

In some embodiments, the cultured cells produce a heterologous polypeptide. In some embodiments, the heterologous polypeptide is an antibody.

In some embodiments, the lactate synthesis rate of the cultured cells is lower than the lactate consumption rate. In some embodiments, the average lactate production rate is less than about negative 0.02 mg/10^6 cells/day.

In some embodiments, the cultured cells containing siRNAs specific for the LDH and PDHK(s) has an osmolality at less than about 300 mOsm.

In some embodiments, the cultured cells have a Specific Productivity (Qp) of at least about 75% higher than cultured cells without the heterologous nucleic acid sequence comprising the PDHK(s) and the LDH.

In some embodiments, the cultured cells have a Specific Productivity (Qp) of at least about 75% higher than cultured cells without the siRNAs specific for the LDH and PDHK(s).

In some embodiments, the cultured cells have a polypeptide productivity (e.g., antibody productivity or titer in g/L) of about 10% to about 800% higher than cultured cells without the heterologous nucleic acid sequence comprising the PDHK(s) and the LDH. In some embodiments, the cultured cells have a polypeptide productivity of about 55% higher than cultured cells without the heterologous nucleic acid sequence comprising the PDHK(s) and the LDH. In some embodiments, the cultured cells have of a polypeptide productivity of at least about 68% higher than cultured cells without the heterologous nucleic acid sequence comprising the PDHK(s) and the LDH.

In some embodiments, the cultured cells have a polypeptide productivity of about 10% to about 800% higher than cultured cells without the siRNAs specific for the PDHK(s) and the LDH. In some embodiments, the cultured cells have a polypeptide productivity of about 55% higher than cultured cells without the siRNAs specific for the PDHK(s) and the LDH. In some embodiments, the cultured cells have a polypeptide productivity of at least about 68% higher than cultured cells without the siRNAs specific for the PDHK(s) and the LDH.

In some embodiments, the cultured cells are mammalian cells. In some embodiments, the cultured cells are non-mammalian cells.

In another aspect, the invention provides a method of silencing or down-regulating LDH and PDHK transcription in a cultured cell comprising: introducing into the cell a vector comprising a first heterologous nucleic acid sequence encoding a siRNA specific for the LDH and a second heterologous nucleic acid sequence encoding an siRNA specific for the PDHK, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter, wherein the siRNAs are expressed, thereby silencing or down-regulating gene transcription of the LDH and the PDHK.

In another aspect, the invention provides a method of making a cell that exhibits decreased lactate production in culture, comprising introducing into the cell a vector comprising a first heterologous nucleic acid sequence encoding a siRNA specific for the LDH and a second heterologous nucleic acid sequence encoding an siRNA specific for the PDHK, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter.

In another aspect, the invention provides a vector comprising a first heterologous nucleic acid sequence encoding a small interfering RNA (siRNA) specific for a lactate dehydrogenase (LDH) and a second heterologous nucleic acid sequence encoding an siRNA specific for a pyruvate dehydrogenase kinase (PDHK), wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows siRNA construct targeting LDHa/PDHK1, 2, 3. siRNAs targeting LDHa, PDHK1, PDHK2 and PDHK3 were cloned into single pSilencer 3.1 hygromycin vector. Targeting sequence for LDHa was under U6 promoter regulation whereas siRNAs for PDHK1, 2, and 3 were under H1 promoter regulation.

FIG. **2** shows relative LDHa, PDHK1, 2, and 3 mRNA expression levels in selected 12 siRNA clones (as shown in light gray color). Expression levels of LDHa and PDHKs were normalized to housekeeping gene b-microglobulin. The average mRNA expression levels from 12 mock clones were shown in dark gray color.

FIG. **3** shows lactate profiles, average lactate production rates, and day 14 pH values in fed-batch shake flask evaluation. Lactate concentrations were measured using Nova analyzer on day 3, 7, 10 and 14 during a 14-day shake flask evaluation. **3**A). Lactate profile of mock (dark gray) and siRNA (light gray) clones; **3**B). Average lactate production rate between days 3 and 14 (mg/10^6 cells/day); and **3**C). Day 14 pH values. The fed-batch shake flask experiments were performed 3 times and the data shown is from 1 experiment.

FIG. **4** shows titer, Specific Productivity (Qp) and cell growth profiles in fed-batch shake flask evaluation. **4**A). Day 14 titer (productivity) in g/L; **4**B). Specific Productivity in pg/cell/day; and **4**C). Cell growth measure by integrated viable cell count (IVCC) in 100 millions of cells per day per liter. Mock clones are in dark gray and siRNA clones are in light gray.

FIG. **5** shows lactate profile, average lactate production rates, and osmolality profile in 2 L bioreactor evaluations. **5**A). Lactate profile; **5**B). Average lactate production rates; and **5**C). Osmolality profile.

FIG. **6** shows productivity profile of cultured cells containing siRNA, mock, or parent clones in 2 L bioreactor evaluation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for reducing lactate production and increasing polypeptide production in cultured cells. The inventors have discovered that concomitant downregulation of a LDH and PDHKs via siRNAs by a process known as RNA interference (RNAi) in cultured cells expressing polypeptides (e.g., antibodies) decreased lactate level, lactate production rate, and cell osmolality, and increased specific polypeptide productivity (e.g., Specific Productivity) and polypeptide production (e.g., productivity). Further, these cultured cells with the downregulated LDH and PDHKs exhibited no negative impact on cell growth, cell viabilities, and the quality of polypeptides produced. Thus, without wishing to be bound by theory, decreasing the pyruvate-lactate conversion by knocking down the expression of a LDH and promoting pyruvate into tricarboxylic acid cycle (TCA or Krebs cycle) by knocking down the expression of one or more PDHKs may create a synergistic effect in lactate reduction and providing cells with more energy and metabolic intermediates. These effects in turn may lead to increased polypeptide (e.g., antibody) production in cultured cells.

Accordingly, in one aspect of the invention, provided is a method for reducing lactate production in cultured cells, comprising culturing cells expressing a) an siRNA specific for a LDH and b) an siRNA specific for a PDHK.

In another aspect, provided are cells in culture comprising a) an si RNA specific for a LDH and an siRNA specific for a PDHK.

In another aspect, the invention provides a method for reducing lactate production in cultured cells, comprising culturing cells comprising a first heterologous nucleic acid sequence encoding an siRNA specific for a LDH and a second heterologous nucleic acid sequence encoding an siRNA specific for a PDHK, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter.

In yet another aspect, the invention provides cells in culture comprising a first heterologous nucleic acid sequence encoding a first siRNA specific for a LDH and a second heterologous nucleic acid sequence encoding a second siRNA specific for a PDHK, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; Oligonucleotide Synthesis (M. J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J. E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R. I. Freshney, ed., 1987); Introduction to Cell and Tissue Culture (J. P. Mather and P. E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J. B. Griffiths, and D. G. Newell, eds., 1993-1998) J. Wiley and

Sons; Methods in Enzymology (Academic Press, Inc.); Handbook of Experimental Immunology (D. M. Weir and C. C. Blackwell, eds.); Gene Transfer Vectors for Mammalian Cells (J. M. Miller and M. P. Calos, eds., 1987); Current Protocols in Molecular Biology (F. M. Ausubel et al., eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Current Protocols in Immunology (J. E. Coligan et al., eds., 1991); Short Protocols in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C. A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: a practical approach (D. Catty., ed., IRL Press, 1988-1989); Monoclonal antibodies: a practical approach (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); Using antibodies: a laboratory manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995).

Definitions

As used herein, the term "cells in culture" or "cultured cells" refers two or more cells in a solution (e.g., a cell medium) that allows the cells to undergo one or more cell divisions.

The term "polynucleotide" or "nucleic acid," as used interchangeably herein, refers to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, cabamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, ply-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, β-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), (O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (—O—) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

The term "RNA interference (RNAi)" refers to the process of sequence-specific, transcriptional gene silencing (e.g., posttranscriptional gene silencing) mediated or initiated by siRNA. Without wishing to be bound by theory, during RNAi, in practicing the methods of the invention, siRNA can induce degradation of target mRNA with consequent sequence-specific inhibition of gene expression of a LDH and one or more PDHKs.

The term "heterologous nucleic acid" or "heterologous polypeptide" refers to a nucleic acid or a polypeptide whose sequence is not identical to that of another nucleic acid or polypeptide naturally found in the same host cell.

The term "small interfering RNA," "short interfering RNA," or "siRNA" refers to an RNA duplex of nucleotides, or, in some alternative aspects, a single molecule of RNA that is targeted to a nucleic acid of interest, e.g., a LDH or PDHK(s). The siRNA comprise a sense RNA strand and a complementary antisense RNA strand annealed together by standard Watson-Crick base-pairing interactions. SiRNA can either be transfected directly or otherwise produced within a cultured cell.

In one variation, the sense RNA strand and the complementary antisense RNA strand are linked by a spacer leading to the expression of a stem-loop or a hairpin structure termed short hairpin RNA (shRNA). The hairpin is then cleaved by an endonuclease (e.g., Dicer) to generate an siRNA. In another variation, the shRNA is a bi-functional shRNA consisting of two stem-loop structures, with one stem-loop structure composed of fully matched sequence guiding the RNA duplex for mRNA degradation via cleavage dependent RISC(RNA-induced silencing complex) loading, and with the second stem-loop structure composed of mis-matched strand inhibiting translation of the mRNA through mRNA sequestration via cleavage-independent RISC loading.

As used herein, an siRNA "specific" for a LDH or PDHK refers to an siRNA that is targeted to a nucleic acid of interest (e.g., a LDH or PDHK(s)) and that the nucleotide sequence of the duplex portion of the siRNA is complementary to a nucleotide sequence of the targeted gene (e.g., a LDH or PDHK(s)).

As used herein, "operably linked" as used herein refers to a functional relationship between two or more nucleic acid (e.g., DNA) segments. Typically, it refers to the functional relationship of transcriptional regulatory sequence to a transcribed sequence. For example, a promoter is operably linked to a coding sequence, such as a nucleic acid of the invention, if it stimulates or modulates the transcription of the coding sequence in an appropriate host cell or other expression system. Generally, promoter transcriptional regulatory sequences that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, i.e., they are cis-acting. However, some transcriptional regulatory sequences, such as enhancers, need not be physically contiguous or located in close proximity to the coding sequences whose transcription they enhance.

As used herein, the term "promoter" includes all sequences capable of driving transcription of a coding

sequence in a cultured cell, e.g., a mammalian cell. Thus, promoters used in the constructs of the invention include cis-acting transcriptional control elements and regulatory sequences that are involved in regulating or modulating the timing and/or rate of transcription of a gene (e.g., a LDH or PDHK(s)). For example, a promoter can be a cis-acting transcriptional control element, including an enhancer, a promoter, a transcription terminator, an origin of replication, a chromosomal integration sequence, 5' and 3' untranslated regions, or an intronic sequence, which are involved in transcriptional regulation. These cis-acting sequences typically interact with proteins or other biomolecules to carry out (turn on/off, regulate, modulate, etc.) transcription. "Constitutive" promoters are those that drive expression continuously under most environmental conditions and states of development or cell differentiation. "Inducible" or "regulatable" promoters direct expression of the nucleic acid of the invention under the influence of environmental conditions or developmental conditions. Examples of environmental conditions that may affect transcription by inducible promoters include anaerobic conditions, elevated temperature, drought, or the presence of light.

As used herein, "vector" means a construct, which is capable of delivering, and preferably expressing, one or more gene(s) or sequence(s) of interest (e.g., LDHa and PDHK(s)) in a host cell. Examples of vectors include, but are not limited to, viral vectors, naked DNA or RNA expression vectors, plasmid, cosmid or phage vectors, DNA or RNA expression vectors associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells, such as producer cells. Suitable vectors are those which are compatible with the host cell employed. Suitable vectors can be derived, for example, from a bacterium, a virus (such as bacteriophage T7 or a M-13 derived phage), a cosmid, a yeast, or a plant. Protocols for obtaining and using such vectors are known to those in the art (see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989).

As used herein, the average lactate production rate is calculated as lactate synthesis rate minus lactate consumption rate in mg/cells/day.

As used herein, "Specific Productivity" or "Qp" refers to the specific protein, e.g., antibody, production rate in pg/cell/day. Specific productivity is calculated as protein titer (pg/cell/day)/IVCC (calculate integrated viable cell count; cell/day).

The terms "polypeptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; single-chain antibody molecules; diabodies; linear antibodies; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al, *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., *Nature* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.* 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (i.e. residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (i.e. residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which hypervariable region residues of the recipient are replaced by hypervariable region residues from a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immuno-

globulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the "binding domain" of a heterologous "adhesin" protein (e.g. a receptor, ligand or enzyme) with the effector functions of an immunoglobulin constant domain. Structurally, the immunoadhesins comprise a fusion of the adhesin amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site (antigen combining site) of an antibody (i.e. is "heterologous") and an immunoglobulin constant domain sequence. The immunoglobulin constant domain sequence in the immunoadhesin is preferably derived from γ1, γ2, or γ4 heavy chains since immunoadhesins comprising these regions can be purified by Protein A chromatography (Lindmark et al., *J. Immunol. Meth.* 62:1-13 (1983)).

The term "ligand binding domain" as used herein refers to any native cell-surface receptor or any region or derivative thereof retaining at least a qualitative ligand binding of a corresponding native receptor. In a specific embodiment, the receptor is from a cell-surface polypeptide having an extracellular domain which is homologous to a member of the immunoglobulin supergenefamily. Other receptors, which are not members of the immunoglobulin supergenefamily but are nonetheless specifically covered by this definition, are receptors for cytokines, and in particular receptors with tyrosine kinase activity (receptor tyrosine kinases), members of the hematopoietin and nerve growth factor receptor superfamilies, and cell adhesion molecules, e.g. (E-, L- and P-) selectins.

The term "receptor binding domain" is used to designate any native ligand for a receptor, including cell adhesion molecules, or any region or derivative of such native ligand retaining at least a qualitative receptor binding ability of a corresponding native ligand. This definition, among others, specifically includes binding sequences from ligands for the above-mentioned receptors.

An "antibody-immunoadhesin chimera" comprises a molecule which combines at least one binding domain of an antibody (as herein defined) with at least one immunoadhesin (as defined in this application). Exemplary antibody-immunoadhesin chimeras are the bispecific CD4-IgG chimeras described in Berg et al., *PNAS* (*USA*) 88:4723-4727 (1991) and Chamow et al., *J. Immunol.* 153:4268 (1994).

The term "osmolality" refers to the number of solute particles dissolved in 1 liter of solution. Solutes which can be added to the culture medium so as to increase the osmolality thereof include proteins, peptides, amino acids, non-metabolized polymers, vitamins, ions, salts (e.g., sodium or potassium salts), sugars, metabolites, organic acids, lipids, etc. When used herein, the abbreviation "mOsm" means "milliosmoles/Liter H_2O."

As used herein, a "host cell" includes an individual cell, cultured cells, or cell in culture that can be or has been a recipient for vector(s) or siRNA(s) for incorporation of polynucleotide inserts to produce polypeptide. Host cells include progeny of a single cultured cell, and the progeny may not necessarily be completely identical (in morphology or in genomic DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation.

For use herein, unless clearly indicated otherwise, use of the terms "a", "an," and the like refers to one or more.

Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X." Numeric ranges are inclusive of the numbers defining the range.

It is understood that wherever embodiments are described herein with the language "comprising," otherwise analogous embodiments described in terms of "consisting of" and/or "consisting essentially of" are also provided.

Where aspects or embodiments of the invention are described in terms of a Markush group or other grouping of alternatives, the present invention encompasses not only the entire group listed as a whole, but each member of the group individually and all possible subgroups of the main group, but also the main group absent one or more of the group members. The present invention also envisages the explicit exclusion of one or more of any of the group members in the claimed invention.

Methods for Reducing Lactate Production

The methods herein involve culturing cells expressing siRNAs specific for a LDH and at least one or more PDHKs to reduce lactate production via RNA interference (RNAi). In one aspect, the method comprises culturing cells expressing a) an siRNA specific for LDH and b) an siRNA specific for a PDHK.

In some embodiments, the cultured cells further express an siRNA specific for a second PDHK. In some embodiments, the cultured cells further express an siRNA specific for a third PDHK. In some embodiments, the cultured cells further express an siRNA specific for a fourth PDHK.

In another aspect, the method comprises a first heterologous nucleic acid sequence encoding an siRNA specific for a LDH and a second heterologous nucleic acid sequence encoding an siRNA specific for a PDHK, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter.

In another aspect, provided is a method of silencing or down-regulating LDH and PDHK transcription in a cultured cell comprising: introducing into the cell a vector comprising a first heterologous nucleic acid sequence encoding an siRNA specific for the LDH and a second heterologous nucleic acid sequence encoding an siRNA specific for the PDHK, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter, wherein the siRNAs are expressed, thereby silencing or down-regulating gene transcription of the LDH and the PDHK.

In some embodiments, the cultured cells further comprise a third heterologous nucleic acid sequence encoding an siRNA specific for a second PDHK and wherein the third heterologous nucleic acid sequence is operably linked to a third promoter. In some embodiments, the cultured cells further comprise a fourth heterologous nucleic acid sequence encoding an siRNA specific for a third PDHK and wherein the fourth heterologous nucleic acid sequence is

operably linked to a fourth promoter. In some embodiments, the cultured cells further comprise a fifth heterologous nucleic acid sequence encoding an siRNA specific for a fifth PDHK and wherein the fifth heterologous nucleic acid sequence is operably linked to a fifth promoter.

In some embodiments, the LDH is LDHa, LDHb, or LDHc. In some embodiments, the PDHK is selected from the group consisting of PDHK1, PDHK2, PDHK3, and PDHK4. In some embodiments, the PDHK is selected from the group consisting of PDHK1, PDHK2, and PDHK3. In some embodiments, the PDHK is selected from the group consisting of PDHK2, PDHK3, and PDHK4. In some embodiments, the PDHK is selected from the group consisting of PDHK1, PDHK3, and PDHK4. In some embodiments, the PDHK is selected from the group consisting of PDHK1 and PDHK2. In some embodiments, the PDHK is selected from the group consisting of PDHK1 and PDHK3. In some embodiments, the PDHK is selected from the group consisting of PDHK2 and PDHK3. In some embodiments, the PDHK is selected from the group consisting of PDHK2 and PDHK4. In some embodiments, the PDHK is selected from the group consisting of PDHK3 and PDHK4.

In some embodiments, the method comprises culturing cells expressing a) an siRNA specific for LDHa and b) an siRNA specific for PDHK1, PDHK2, and PDHK3, respectively. In some embodiments, the method comprises culturing cells expressing a) an siRNA specific for LDHb and b) an siRNA specific for PDHK1, PDHK2, and PDHK3, respectively. In some embodiments, the method comprises culturing cells expressing a) an siRNA specific for LDHc and b) an siRNA specific for PDHK1, PDHK2, and PDHK3, respectively.

In some embodiments, the method comprises culturing cells expressing a) an siRNA specific for LDHa, LDHb, or LDHc and b) an siRNA specific for two PDHKs, wherein the PDHK is selected from the group consisting of PDHK1, PDHK2, PDHK3, and PDHK4. For example, the method comprises culturing cells expressing a) an siRNA specific for LDHa and b) an siRNA specific for PDHK1 and PDHK2, respectively.

In some embodiments, the mRNA expression level for a LDH is reduced by at least about 75% and the mRNA expression level for a PDHK is reduced by at least about 25% in cultured cells expressing a) an siRNA specific for a LDH and b) an siRNA specific for a PDHK in comparison to cultured cells without the siRNAs specific for a LDH and a PDHK. In some embodiments, the LDH is LDHa, LDHb, or LDHc and the mRNA expression level for the LDH is reduced by at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%. In some embodiments, the PDHK is PDHK1, PDHK2, or PDHK3, and the mRNA expression level for the PDHK is reduced by at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%.

In some embodiments, in cultured cells expressing a) an siRNA specific for LDHa and b) an siRNA specific for PDHK1, PDHK2, and PDHK3, the mRNA expression level for LDHa is reduced by about 90% and the mRNA expression levels for PDHK1, PDHK2, and PDHK3 are reduced by about 32%, 83%, and 70%, respectively, in comparison to cultured cells without the siRNAs specific for the LDHa, PDHK1, PDHK2, and PDHK3.

In some embodiments, the method comprises a first heterologous nucleic acid sequence encoding an siRNA specific for LDHa, LDHb, or LDHc, a second heterologous nucleic acid sequence encoding an siRNA specific for PDHK1, a third heterologous nucleic acid sequence encoding an siRNA specific for PDHK2, and a fourth heterologous nucleic acid sequence encoding an siRNA specific for PDHK3, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second, third, and fourth heterologous nucleic acid sequence is operably linked to a second promoter.

In some embodiments, the method comprises a first heterologous nucleic acid sequence encoding an siRNA specific for LDHa, LDHb, or LDHc, a second heterologous nucleic acid sequence encoding an siRNA specific for a PDHK, and a third heterologous nucleic acid sequence encoding an siRNA specific for a PDHK, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, wherein the second and third heterologous nucleic acid sequences are operably linked to a second promoter, and wherein the PDHK is selected from the group consisting of PDHK1, PDHK2, PDHK3, and PDHK4.

In some embodiments, the mRNA expression level for a LDH is reduced by at least about 75% and the mRNA expression level for a PDHK is reduced by at least about 25% in cultured cells comprising a first heterologous nucleic acid sequence encoding an siRNA specific for a LDH and a second heterologous nucleic acid sequence encoding an siRNA specific for a PDHK in comparison to cultured cells without the heterologous nucleic acid sequence comprising the LDH and the PDHK(s), wherein the first heterologous nuclei acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter. In some embodiments, the LDH is LDHa, LDHb, or LDHc and the mRNA expression level for the LDH is reduced by at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%. In some embodiments, the PDHK is PDHK1, PDHK2, or PDHK3, and the mRNA expression level for the PDHK is reduced by at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%.

In some embodiments, in cultured cells comprising a first heterologous nucleic acid sequence encoding an siRNA specific for LDHa, a second heterologous nucleic acid sequence encoding an siRNA specific for PDHK1, a third heterologous nucleic acid sequence encoding an siRNA specific for PDHK2, and a fourth heterologous nucleic acid sequence encoding an siRNA specific for PDHK3, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second, third, and fourth heterologous nucleic acid sequence is operably linked to a second promoter, the mRNA expression level for LDHa is reduced by about 90% and the mRNA expression levels for PDHK1, PDHK2, and PDHK3 are reduced by about 32%, 83%, and 70%, respectively, in comparison to cultured cells without the siRNAs specific for the LDHa, PDHK1, PDHK2, and PDHK3.

The siRNA used in the invention described herein can be obtained or made from a variety of sources, e.g., produced in vitro, ex vivo or in vivo, as described herein. In some embodiments, the siRNA can contain from about 1 to about 200 nucleotides, from about 5 to about 100 nucleotides, from about 10 to about 50 nucleotides, from about 15 to

about 30 nucleotides, or from about 19 to about 25 nucleotides. In some embodiments, the length of the siRNA is less than 30 nucleotides. In some embodiments, the length of the siRNAs is more than 30 nucleotides. In some embodiments, the siRNA can be 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9 or less nucleotides in length.

In some embodiments, the siRNA can be generated by chemical synthesis, by in vitro transcription using a polymerase, or by an endoribonuclease (e.g., Dicer) digestion of long double strand RNA (dsRNA). In some embodiments, the siRNA can be entirely, or in part, comprised of synthetic nucleotides, natural bases or modified bases.

In some embodiments, the siRNA can be expressed intracellularly. The siRNA can be encoded by a nucleic acid sequence, and the nucleic acid sequence can also include one or more promoters. The nucleic acid sequence can also include a polyadenylation signal. In some embodiments, sense and antisense strands of the RNA duplex can be produced from two independent promoters and annealed with the cultured cell. In some embodiments, the sense and antisense strands of the RNA duplex can also be linked by a base pair spacer (e.g., a base pair spacer may comprise a single or multiple base pair) or a stem-loop to form a shRNA and expressed by a single promoter. In some embodiments, the shRNA can be a bi-functional shRNA. The hairpin can be cleaved by an endoribonuclease (e.g., Dicer) to generate effective siRNA molecules. The spacer or stem-loop is positioned between the sense and antisense strands that form the duplex. The stem-loop can vary in length. In some embodiments, the stem-loop is 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 or more nucleotides in length. The hairpin structure can also contain 3' or 5' overhang portions. In some embodiments, the overhang is a 3' or a 5' overhang 1, 2, 3, 4 or 5 nucleotides in length. Compositions and methods for RNA-mediated gene regulation by siRNA, shRNA, or bifunctional shRNA are described, for example, in U.S. Appl. No. 20090215860, Rutz and Scheffold, *Arthritis Research & Therapy,* 6(2):78-85 (2004), and Rao et al., *Advanced Drug Delivery Reviews* 61:746-759 (2009).

In some embodiments, the siRNA used in the present invention can have perfect homology with target sequences to produce target specific responses. In some embodiments, the siRNA used in the present invention have about any of 99%, 98%, 97%, 96%, 95%, 94%, 92%, 91%, 90%, 88%, 86%, 84%, 82%, 80%, 78%, 76%, 74%, 72%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, or 5%, homology with target sequences. In one variation, the siRNA used in the present invention can hybridize under physiologic conditions to a nucleic acid target sequence, e.g., it can specifically hybridize to a target sequence in a cell, e.g., in vivo. In another variation, the siRNA targets more than one target sequence, target marker or reporter gene.

The extent of sequence identity (homology) necessary for in vivo targeting of an siRNA to a target nucleic acid (e.g., specific binding of an siRNA to a target sequence in a cell under physiologic conditions) can be tested under routine screening conditions, e.g., in cell culture and the like.

In some embodiments, the target sequence for PDHK1 is GCAGTTCCTGGACTTCGGA (SEQ ID NO:2). In some embodiments, the target sequence for PDHK2 is CATTCAGTACTTCTTGGAC (SEQ ID NO:3). In some embodiments, the target sequence for PDHK3 is TGTAGCTGATGTCGTGAAA (SEQ ID NO:4).

Lactate dehydrogenase (LDH) converts pyruvate into lactate. The accession numbers of exemplary LDH (e.g., LDHa, LDHb, or LDHc) polypeptides and nucleic acids include, but are not limited to, DQ912661 (LDHa in CHO cells), BC067223 (human LDHa), BC084698 (rat LDHa), BC094428 (mouse LDHa), BC002362 (human LDHb), NM_012595 (rat LDHb), NM_008492 (mouse LDHb), BC090043 (human LDHc), NM_017266 (rat LDHc), and NM_013580 (mouse LDHc). Standard methods known by persons skill in the art can be used to determine whether a LDH polypeptide has LDH activity by measuring the ability of the polypeptide to convert pyruvate into lactate in vitro, in a cell extract, or in vivo.

Pryuvate dehydrogense kinase (PDHK) inhibits the conversion of pyruvate into acetyl-CoA. The accession numbers of exemplary PDHK1 polypeptides and nucleic acids include, but are not limited to, L42450 (human), BC089783 (rat), and NM_172665 (mouse). The accession numbers of exemplary PDHK2 polypeptides and nucleic acids include, but are not limited to, NM_002611 (human), NM_030872 (rat), and NM_133667 (mouse). The accession numbers of exemplary PDHK3 polypeptides and nucleic acids include, but are not limited to, L42452 (human), BC169078 (rat), and NM_145630 (mouse). The accession numbers of exemplary PDHK4 polypeptides and nucleic acids include, but are not limited to, NM_002612 (human), NM_053551 (rat), and NM_013743 (mouse). Standard methods known by person skilled in the art can be used to determine whether a PDHK polypeptide has PDHK activity by measuring the ability of the polypeptide to inhibit the conversion of pyruvate into acetyl-CoA in vitro, in a cell extract, or in vivo.

Promoters are well known in the art. Any promoter that functions in the host cell can be used for expression of siRNAs specific for a LDH and one or more of PDHK in the host cell. Virtually any promoter capable of driving these siRNAs is suitable for the present invention including, but not limited to, U6, H1, CYC1, HIS3, GAL1, GAL4, GAL10, ADH1, PGK, PHO5, GAPDH, T7, CMV, SV40, and EF1a. For example, in some embodiments, the method comprises a first heterologous nucleic acid sequence encoding an siRNA specific for LDHa, a second heterologous nucleic acid sequence encoding an siRNA specific for PDHK1, a third heterologous nucleic acid sequence encoding an siRNA specific for PDHK2, and a fourth heterologous nucleic acid sequence encoding an siRNA specific for PDHK3, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter U6, and wherein the second, third, and fourth heterologous nucleic acid sequences are operably linked to a second promoter H1. In one variation, the first heterologous nucleic acid sequence encoding an siRNA is specific for LDHb. In another variation, the first heterologous nucleic acid sequence encoding an siRNA is specific for LDHc.

In another aspect, provided is a method of making a cell that exhibits decreased lactate production in culture, comprising introducing into the cell a vector comprising a first heterologous nucleic acid sequence encoding an siRNA specific for the LDH and a second heterologous nucleic acid sequence encoding an siRNA specific for the PDHK, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter.

The first heterologous nucleic acid sequence encoding an siRNA specific for the LDH and the second heterologous nucleic acid sequence encoding the siRNA specific for the PDHK can be inserted into a vector by a variety of procedures. For example, the LDH and PDHK siRNA sequences are ligated to the desired position in the vector following

digestion of the insert and the vector with appropriate restriction endonucleases, such as KasI, BamHI, HindIII, or BhIII. In some embodiments, a vector containing siRNAs sequences specific for LDHa and PDHK1, PDHK2, and PDHK3 are constructed by inserting the LDHa siRNA sequence into the KasI site of the vector (e.g., pSilencer 3.1-H1 hygro vector) with an addition of U6 promoter at its immediate 5' end, inserting the PDHK1 and PDHK2 siRNA sequences into BamHI/HindIII and HindIII sites, respectively, and inserting the PDHK3 siRNA sequence into BgIII with an addition of H1 promoter at the immediate 5' ends of PDHK1, PDHK2, and PDHK3. Cultured cells expressing decreased lactate production can then be generated by transfecting the vectors containing LDHa and PDHK1, PDHK2, and PDHK3 siRNA.

Compositions

The cultured cells produced by the methods described herein are also provided in the present invention. The compositions of the present invention can be practiced in vivo, ex vivo, or in vitro. In one aspect, provided are cells in culture expressing a) an siRNA specific for LDH and b) an siRNA specific for a PDHK. In some embodiments, the cultured cells further express an siRNA specific for a second PDHK. In some embodiments, the cultured cells further express an siRNA specific for a third PDHK. In some embodiments, the cultured cells further express an siRNA specific for a fourth PDHK.

In some embodiments, cells in culture express a) an siRNA specific for LDHa and b) an siRNA specific for PDHK1, PDHK2, and PDHK3, respectively. In some embodiments, cells in culture express a) an siRNA specific for LDHb and b) an siRNA specific for PDHK1, PDHK2, and PDHK3, respectively. In some embodiments, cells in culture express a) an siRNA specific for LDHc and b) an siRNA specific for PDHK1, PDHK2, and PDHK3, respectively.

In some embodiments, cells in culture express a) an siRNA specific for LDHa and b) an siRNA specific for two PDHKs, wherein the PDHK is selected from the group consisting of PDHK1, PDHK2, PDHK3, and PDHK4. In some embodiments, cells in culture express a) an siRNA specific for LDHb and b) an siRNA specific for two PDHKs, wherein the PDHK is selected from the group consisting of PDHK1, PDHK2, PDHK3, and PDHK4. In some embodiments, cells in culture express a) an siRNA specific for LDHc and b) an siRNA specific for two PDHKs, wherein the PDHK is selected from the group consisting of PDHK1, PDHK2, PDHK3, and PDHK4.

In another aspect, provided are cells in culture comprising a first heterologous nucleic acid sequence encoding an siRNA specific for a LDH and a second heterologous nucleic acid sequence encoding an siRNA specific for a PDHK, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter. In some embodiments, the cells further comprise a third heterologous nucleic acid sequence encoding an siRNA specific for a second PDHK and wherein the third heterologous nucleic acid sequence is operably linked to a third promoter. In some embodiments, the cells further comprise a fourth heterologous nucleic acid sequence encoding an siRNA specific for a third PDHK and wherein the fourth heterologous nucleic acid sequence is operably linked to a fourth promoter. In some embodiments, the cells further comprise a fifth heterologous nucleic acid sequence encoding an siRNA specific for a fifth PDHK and wherein the fifth heterologous nucleic acid sequence is operably linked to a fifth promoter.

In some embodiments, cells in culture comprises a first heterologous nucleic acid sequence encoding an siRNA specific for LDHa, a second heterologous nucleic acid sequence encoding an siRNA specific for PDHK1, a third heterologous nucleic acid sequence encoding an siRNA specific for PDHK2, and a fourth heterologous nucleic acid sequence encoding an siRNA specific for PDHK3, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter (e.g., U6), and wherein the second, third, and fourth heterologous nucleic acid sequences are operably linked to a second promoter (e.g., H1). In one variation, the first heterologous nucleic acid sequence encoding an siRNA is specific for LDHb. In another variation, the first heterologous nucleic acid sequence encoding an siRNA is specific for LDHb.

In some embodiments, cells in culture comprise a first heterologous nucleic acid sequence encoding an siRNA specific for LDHa, a second heterologous nucleic acid sequence encoding an siRNA specific for a PDHK, a third heterologous nucleic acid sequence encoding an siRNA specific for a PDHK, wherein the PDHK is selected from the group consisting of PDHK1, PDHK2, PDHK3, and PDHK4, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter (e.g., U6), and wherein the second and the third heterologous nucleic acid sequences are operably linked to a second promoter (e.g., H1). In one variation, the first heterologous nucleic acid sequence encoding an siRNA is specific for LDHb. In another variation, the first heterologous nucleic acid sequence encoding an siRNA is specific for LDHc.

In some embodiments, the cell culture includes at least about 5, 10, 15, 20, 50, 75, 100, 200, 500, 750, 1,000, 5,000, 7,500, 10,000, 15,000 or more cells.

In another aspect, provided are cells in culture having a lactate synthesis rate that is lower than a lactate consumption rate. In some embodiments, the cells in culture have an average lactate production rate of less than about any of negative 0.2 mg/10^6 cells/day, negative 0.1 mg/10^6 cells/day, negative 0.08 mg/10^6 cells/day, negative 0.06 mg/10^6 cells/day, negative 0.04 mg/10^6 cells/day, negative 0.02 mg/10^6 cells/day, negative 0.01 mg/10^6 cells/day, negative 0.008 mg/10^6 cells/day, negative 0.006 mg/10^6 cells/day, negative 0.004 mg/10^6 cells/day, or negative 0.002 mg/10^6 cells/day.

In some embodiments, cells in culture comprises a first heterologous nucleic acid sequence encoding an siRNA specific for LDHa, a second heterologous nucleic acid sequence encoding an siRNA specific for PDHK1, a third heterologous nucleic acid sequence encoding an siRNA specific for PDHK2, and a fourth heterologous nucleic acid sequence encoding an siRNA specific for PDHK3, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter (e.g., U6), wherein the second, third, and fourth heterologous nucleic acid sequences are operably linked to a second promoter (e.g., HD, and wherein the cells in culture have an average lactate production rate of about negative 0.02 mg/10^6 cells/day.

In another aspect, provided are cells in culture containing siRNA specific for a LDH and PDHK(s) having a decreased osmolality. In some embodiments, cells in culture containing siRNA specific for a LDH and PDHK(s) have an osmolality at less than about any of 500 mOsm, 450 mOsm, 400 mOsm 350 mOsm, 300 mOsm, 250 mOsm, 200 mOsm, or 150 mOsm.

In some embodiments, cells in culture comprises a first heterologous nucleic acid sequence encoding an siRNA specific for LDHa, a second heterologous nucleic acid sequence encoding an siRNA specific for PDHK1, a third heterologous nucleic acid sequence encoding an siRNA specific for PDHK2, and a fourth heterologous nucleic acid sequence encoding an siRNA specific for PDHK3, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter (e.g., U6), wherein the second, third, and fourth heterologous nucleic acid sequence is operably linked to a second promoter (e.g., HD, and wherein the cells in culture have an osmolality at about 300 mOsm.

In another aspect, provided are cells in culture having an increased Specific Productivity (Qp). In some embodiments, the cultured cells have a Specific Productivity of at least about 60% higher, at least about 65% higher, at least about 70% higher, at least about 75% higher, at least about 80% higher, at least about 85% higher, at least about 90% higher, or at least about 95% higher than cultured cells without the heterologous nucleic acid sequence comprising the PDHK(s) and the LDH. In some embodiments, the cultured cells have a Specific Productivity of about 67% higher, about 69% higher, about 71% higher, about 72% higher, about 73% higher, about 74% higher, about 75% higher, about 76% higher, about 77% higher, about 78% higher, about 79% higher, about 81% higher, about 83% higher, about 85% higher, about 87% higher, about 89% higher, about 91% higher, about 93% higher, about 95% higher, about 97% higher, or at about 99% higher than cultured cells without the heterologous nucleic acid sequence comprising the PDHK(s) and the LDH.

In some embodiments, cells in culture comprises a first heterologous nucleic acid sequence encoding an siRNA specific for LDHa, a second heterologous nucleic acid sequence encoding an siRNA specific for PDHK1, a third heterologous nucleic acid sequence encoding an siRNA specific for PDHK2, and a fourth heterologous nucleic acid sequence encoding an siRNA specific for PDHK3, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter (e.g., U6), wherein the second, third, and fourth heterologous nucleic acid sequence is operably linked to a second promoter (e.g., H1), and wherein the cells in culture have a Specific Productivity of about 75% higher.

In another aspect, provided are the cultured cells produced by the method herein with an increased polypeptide productivity (e.g., antibody productivity or titer in g/L). In some embodiments, the cultured cells have a polypeptide productivity of about 10% to about 800% higher than cultured cells without the heterologous nucleic acid sequence comprising the PDHK(s) and the LDH. In some embodiments, the cultured cells have a polypeptide productivity of about 10% higher, about 15% higher, about 20% higher, about 25% higher, about 30% higher, about 35% higher, about 40% higher, about 45% higher, about 50% higher, about 55% higher, about 58% higher, about 60% higher, about 65% higher, about 70% higher, about 71% higher, about 75% higher, about 80% higher, about 85% higher, about 90% higher, about 95% higher, about 100% higher, about 125% higher, about 150%, about 200% higher, about 250% higher, about 300% higher, about 350% higher, about 400% higher, about 450% higher, about 500 higher, about 550% higher, about 600% higher, about 650% higher, about 700% higher, about 750% higher, or about 800% higher than cultured cells without the heterologous nucleic acid sequence comprising the PDHK(s) and the LDH. In some embodiments, the cultured cells have a polypeptide productivity of at least about 55% higher, at least about 60% higher, at least about 65% higher, at least about 68% higher, at least about 70% higher, at least about 80% higher, at least about 85% higher, or at least about 90% higher than cultured cells without the heterologous nucleic acid sequence comprising the PDHK(s) and the LDH.

In some embodiments, cells in culture comprises a first heterologous nucleic acid sequence encoding an siRNA specific for LDHa, a second heterologous nucleic acid sequence encoding an siRNA specific for PDHK1, a third heterologous nucleic acid sequence encoding an siRNA specific for PDHK2, and a fourth heterologous nucleic acid sequence encoding an siRNA specific for PDHK3, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter (e.g., U6), wherein the second, third, and fourth heterologous nucleic acid sequence is operably linked to a second promoter (e.g., H1), and wherein the cultured cells have an antibody productivity (e.g., in g/L) of at least about 68% higher than cultured cells without the heterologous nucleic acid sequence comprising the PDHK1, PDHK2, PDHK3, and LDHa.

In some embodiments, the cultured cells have a polypeptide productivity of about 10% to about 800% higher than cultured cells without the siRNAs specific for the PDHK(s) and the LDH (in some embodiments, an antibody). In some embodiments, the cultured cells have a polypeptide productivity of about 10% higher, about 15% higher, about 20% higher, about 25% higher, about 30% higher, about 35% higher, about 40% higher, about 45% higher, about 50% higher, about 55% higher, about 60% higher, about 65% higher, about 70% higher, about 75% higher, about 80% higher, about 85% higher, about 90% higher, about 95% higher, about 100% higher, about 125% higher, about 150%, about 200% higher, about 250% higher, about 300% higher, about 350% higher, about 400% higher, about 450% higher, about 500 higher, about 550% higher, about 600% higher, about 650% higher, about 700% higher, about 750% higher, or about 800% higher than cultured cells without the siRNAs specific for the PDHK(s) and the LDH. In some embodiments, the cultured cells have a polypeptide productivity of at least about 65% higher, at least about 68% higher, at least about 70% higher, at least about 80% higher, at least about 85% higher, or at least about 90% higher than cultured cells w without the siRNAs specific for the PDHK(s) and the LDH. In some embodiments, the antibody productivity is at least about 68% higher than cultured cells without the siRNAs specific for the PDHK(s) and the LDH.

In another aspect, provided is a vector comprising a first heterologous nucleic acid sequence encoding an siRNA specific for a LDH and a second heterologous nucleic acid sequence encoding an siRNA specific for a PDHK, wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter.

In some embodiments, the vector contains a nucleic acid under the control of an expression control sequence. As used herein, an “expression control sequence” means a nucleic acid sequence that directs transcription of a nucleic acid of interest. An expression control sequence can be a promoter, such as a constitutive or an inducible promoter, or an enhancer. An “inducible promoter” is a promoter that is active under environmental or developmental regulation. The expression control sequence is operably linked to the nucleic acid segment to be transcribed.

In some embodiments, the vector also includes a termination sequence. Termination control regions may also be

derived from various genes native to the host cell. In some embodiments, the termination sequence and the promoter sequence are derived from the same source. In another embodiment, the termination sequence is endogenous to the host cell. Optionally, a termination site may be included. For effective expression of the polypeptides, DNA encoding the polypeptide are linked operably through initiation codons to selected expression control regions such that expression results in the formation of the appropriate messenger RNA.

In some embodiments, the vector contains a selective marker. The term "selective marker" refers to a nucleic acid capable of expression in a host cell that allows for ease of selection of those host cells containing an introduced nucleic acid or vector. Examples of selectable markers include, but are not limited to, antibiotic resistance nucleic acids (e.g., kanamycin, ampicillin, carbenicillin, gentamicin, hygromycin, phleomycin, bleomycin, neomycin, or chloramphenicol) and/or nucleic acids that confer a metabolic advantage, such as a nutritional advantage on the host cell. In some embodiments, the selective marker is the hygromycin nucleic acid.

Polypeptides

The polypeptide or protein to be produced using the methods and cultured cells described herein includes, but is not limited to, antibody or immunoadhesin. Techniques for generating such molecules are discussed below.

Antibodies

Antibodies within the scope of the present invention include, but are not limited to: anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN®); anti-VEGF antibodies, including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors,* 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998) and V3LA; anti-MUC16 antibody; anti-CD4 antibodies such as the cM-7412 antibody (Choy et al. *Arthritis Rheum.* 39(1):52-56 (1996)) and the Ibalizumab (TNX355) antibody; anti-MET antibodies such as one-armed 5D5 anti-C-Met antibody; anti-HER2 antibodies Trastuzumab (HERCEPTIN®) (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285-4289 (1992), U.S. Pat. No. 5,725,856) and humanized 2C4 (WO01/00245, Adams et al.), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108B1, or Tositumomab (BEXXAR®); anti-IL-8 antibodies (St John et al., *Chest,* 103:932 (1993), and International Publication No. WO 95/23865); anti-prostate stem cell antigen (PSCA) antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD1 antibodies (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., *Transplant Intl.* 4:3-7 (1991), and Hourmant et al., *Transplantation* 58:377-380 (1994)); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul. 31, 1997); anti-IgE antibodies (including E25, E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No. 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No. PCT/US98/13410 filed Jun. 30, 1998, U.S. Pat. No. 5,714,338, Presta et al., *J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181); anti-Apo-2 receptor antibodies (WO 98/51793 published Nov. 19, 1998); anti-TNF-α antibodies, including cA2 (REMICADE®), CDP571 and MAK-195 (See, U.S. Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al. *J. Immunol.* 156(4):1646-1653 (1996), and Dhainaut et al. *Crit. Care Med.* 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) antibodies (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human α4β7 integrin antibodies (WO 98/06248 published Feb. 19, 1998); anti-epidermal growth factor receptor (EGFR) antibodies (e.g. chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S. Pat. No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-Tac antibodies such as CHI-621 (SIMULECT® and ZENAPAX® (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al. Nature 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22 antibody directed against Fcγ RI as in Graziano et al. *J. Immunol.* 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-1 4 (Sharkey et al. *Cancer Res.* 55(23Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al. *Cancer Res.* 55(23): 5852s-5856s (1995); and Richman et al. *Cancer Res.* 55(23 Supp): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al. *Eur J Immunol.* 26(1): 1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al. *J. Immunol.* 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al. *Cancer Res* 55(23 Suppl):5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22 antibodies such as LL2 or LymphoCide (Juweid et al. *Cancer Res* 55(23 Suppl):5899s-5907s (1995)); anti-EpCAM antibodies such as 17-1A (PANOREX®); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO®); anti-RSV antibodies such as MEDI-493 (SYNAGIS®); anti-CMV antibodies such as PROTOVIR®; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR®; anti-CA 125 antibodies, such as OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-αvβ3 antibodies, including VITAXIN®; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1).

Aside from the antibodies specifically identified above, the skilled practitioner can generate antibodies directed against an antigen of interest, e.g., using the techniques described below.

(i) Antigen Selection and Preparation

The antibody herein is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those proteins described in section (3) below. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20, CD22 and CD34; members of the ErbB receptor family such as the EGFR, HER^2, HER^3 or HER^4 receptor; cell adhesion molecules such as LFA-1, Mac1, p1 50,95, VLA-4, ICAM-1, VCAM and αv/β3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group anti-

gens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C, or any of the other antigens mentioned herein.

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

(ii) Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to {fraction (1/10)} the original amount of antigen or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(iii) Monoclonal Antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al, *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, Protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. Preferably the Protein A affinity chromatography procedure using a pH gradient described herein is used.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

Monoclonal antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional hybridoma techniques for isolation of monoclonal antibodies.

(iv) Humanized and Human Antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human FR for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immunol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (JH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and Duchosal et al. *Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 (1991); Vaughan et al. *Nature Biotech* 14:309 (1996)).

(v) Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al. *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., *Bio/Technology* 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. A single chain Fv fragment (scFv) can also be isolated. See WO 93/16185. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

(vi) Multispecific Antibodies

Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one

or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g., tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g., alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytic ally cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994). Alternatively, the antibodies can be "linear antibodies" as described in Zapata et al. *Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H and V_L) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. Immunol.* 147: 60 (1991).

Immunoadhesins

The simplest and most straightforward immunoadhesin design combines the binding domain(s) of the adhesin (e.g., the extracellular domain (ECD) of a receptor) with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the immunoadhesins of the present invention, nucleic acid encoding the binding domain of the adhesin will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, C_H2 and C_H3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the C_H1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics of the immunoadhesin.

In some embodiments, the adhesin sequence is fused to the N-terminus of the Fc domain of immunoglobulin G_1 (Ig G_1). It is possible to fuse the entire heavy chain constant region to the adhesin sequence. However, preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (i.e. residue 216, taking the first residue of heavy chain constant region to be 114), or analogous sites of other immunoglobulins is used in the fusion. In some embodiments, the adhesin amino acid sequence is fused to (a) the hinge region and or C_H2 and C_H3 or (b) the C_H1, hinge, C_H2 and C_H3 domains, of an IgG heavy chain.

For bispecific immunoadhesins, the immunoadhesins are assembled as multimers, and particularly as heterodimers or heterotetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of four basic units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each of the four units may be the same or different.

Various exemplary assembled immunoadhesins within the scope herein are schematically diagramed below:

(a) AC_L-AC_L;

(b) AC_H-(AC_H, AC_L-AC_H, AC_L-V_HC_H, or V_LC_L-AC_H);

(c) AC_L-AC_H-(AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H)

(d) AC_L-V_HC_H-(AC_H, or AC_L-V_HC_H, or V_LC_L-AC_H);

(e) V_LC_L-AC_H-(AC_L-V_HC_H, or V_LC_L-AC_H); and

(f) $(A\text{-}Y)_n$-$(V_LC_L\text{-}V_HC_H)_2$,

wherein each A represents identical or different adhesin amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_H is an immunoglobulin heavy chain constant domain;

n is an integer greater than 1;

Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed to be present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the adhesin sequences can be inserted between immunoglobulin heavy chain and light chain sequences, such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the adhesin sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the C_H2 domain, or between the C_H2 and C_H3 domains. Similar constructs have been reported by Hoogenboom, et al., *Mol. Immunol.* 28:1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an adhesin-immunoglobulin heavy chain fusion polypeptide, or directly fused to the adhesin. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the adhesin-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Pat. No. 4,816,567, issued 28 Mar. 1989.

Immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the adhesin portion in-frame to an immunoglobulin cDNA sequence. However, fusion to genomic immunoglobulin fragments can also be used (see, e.g., Aruffo et al., *Cell* 61:1303-1313 (1990); and Stamenkovic et al., *Cell* 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequences from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the "adhesin" and the immunoglobulin parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells.

Expression of Polypeptides

The polypeptide (e.g., antibody) to be produced using the method described herein is generally produced using recombinant techniques.

Suitable host cells for cloning or expressing the siRNAs in the vectors herein are the prokaryote, yeast, or higher eukaryotic cells. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella*, e.g., *Salmonella typhimurium, Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 Apr. 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe; Kluyveromyces* hosts such as, e.g., *K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa; Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., *Neurospora, Penicillium, Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger.*

Suitable cultured cells for the expression of glycosylated polypeptide are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian cell lines include, but are not limited to, monkey kidney CV1 cells transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney cells (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and human hepatoma cells (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the polypeptide used in the methods of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), (Sigma), or GIBCO® Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (Invitrogen) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58:44 (1979), Barnes et al., *Anal. Biochem.* 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. No. Re. 30,985 may be used as culture media for the host cells. Other defined or synthetic growth media may also be used,

and the appropriate medium for growing a specific type of host cells are known by one of skill in the art of molecular and cell biology. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™, hygromycin), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Standard cell culture conditions can be used to culture the cells. Cells are grown and maintained at an appropriate temperature, gas mixture, and pH (such as at about 20° C. to about 37° C., at about 6% to about 84% CO_2, and at a pH between about 5 to about 9). In some embodiments, cells are grown in an appropriate cell medium at 37° C. for the first 48 hours, and shifted to 33° for the next 12 days. Reactions may be performed under aerobic or anoxic conditions based on the requirements of the host cells. In some embodiments, the cells are grown using any known mode of fermentation, including, but not limited to, batch, fed-batch, or continuous processes.

When using recombinant techniques, the polypeptide can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the polypeptide is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells (e.g. resulting from homogenization), is removed, for example, by centrifugation or ultrafiltration. Where the polypeptide is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit.

Kits

The present invention also provides kits comprising compositions and instructions for use comprising description of the methods of the invention. The kits can comprise cultured cells, siRNAs, target sequences, transfecting agents, instructions for the methods of the present invention, or any combination thereof.

The following examples are provided to illustrate, but not to limit, the invention.

EXAMPLES

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

Example 1

Knocking Down of PDHK1, PDHK2, PDHK3, and LDHa Reduces Lactate Production and Increases Antibody Titer/Productivity

Materials and Methods

Construction of the Vector Targeting LDHa/PDHK1, 2, 3

Targeting sequence for LDHa was selected as described previously by Kim and Lee et al, *Appl. Microbiol. Biotechnol.* 74(1):152-159 (2007), and the LDHa siRNA sequence is CTCGATTCCGTTATCTGAT (SEQ ID NO:1). To design the siRNA-targeted sequence for PDHKs, partial cDNA sequences for CHO PDHK1, 2, and 3 were cloned by reverse transcription of polymerase chain reaction (RT-PCR) with primers located within the highly conserved regions of PDHKs. Partially cloned sequences were used for siRNA sequence designing according to the method described by Elbashier et al. (*Methods* 26:199-213 (2002)).

```
PDHK1 targeting (siRNA) sequence:
                                   (SEQ ID NO: 2)
GCAGTTCCTGGACTTCGGA

PDHK2 targeting (siRNA) sequence:
                                   (SEQ ID NO: 3)
CATTCAGTACTTCTTGGAC

PDHK3 targeting (siRNA) sequence:
                                   (SEQ ID NO: 4)
TGTAGCTGATGTCGTGAAA
```

The single construct containing targeting sequences for LDHa and PDHKs was constructed using the pSilencer 3.1-H1 hygro vector (Cat#. AM5766, Applied Biosystems/Ambion, Austin, Tex.). LDHa siRNA was inserted into the KasI site of pSilencer 3.1, with an addition of U6 promoter from pSilencer 2.1 at its immediate 5' end. SiRNA sequences for PDHK1 and 2 siRNAs were inserted into BamHI/HindIII and HindIII sites respectively. A BglII site was introduced to the 3' side of PDHK2 siRNA and used for the insertion of PDHK3 siRNA. For negative control, pSilencer 3.1 vector containing a scrambled siRNA sequence was utilized.

Cell Culture

CHO cells deficient in dihydrofolate reductase (DHFR) were cultured in a proprietary DMEM/F12-based medium in shake flask vessels at 37° C. and 5% CO_2. Cells were passaged every three to four days.

Stable siRNA Cell Line (siRNA Clone) Development

A CHO cell line resistant to 25 nM methotrexate (MTX) and expressing a recombinant monoclonal antibody was transfected using Lipofectamine 2000 CD (Cat#12566-014, Invitrogen, Carlsbad, Calif.) according to manufacturer's recommendation (Invitrogen, Carlsbad, Calif.). Transfected cells were centrifuged and seeded into DMEM/F-12-based selective (glycine-, hypoxanthine- and thymidine-free) medium containing 25 nM MTX and 400 ug/ml hygromycin (Cat #10687010, Invitrogen, Carlsbad, Calif.). Re-suspended cells were plated into 96-well plates to generate individual clones. SiRNA clones were derived from siRNA plasmid transfection containing targeting sequences for LDHa and PDHKs genes, while mock clones were derived from mock plasmid (Cat#AM5766, Applied Biosystems/Ambion, Austin, Tex.) transfection containing a scramble sequence designed by manufacture with no appreciable homology to known genes.

Quantitative Real Time PCR (qRT-PCR or Taqman) Analysis

Total RNA from individual clones were isolated using the RNeasy 96 kit (Cat#74181, Qiagen) and were treated with DNase digestion (Cat#79254, RNase free DNase set, Qiagen) to remove residual DNA possibly present in isolated RNA samples. Taqman was performed using universal qRT-PCR master mix according to the manufacturer's instructions (Cat#4309169, Applied Biosystems) and expression levels of PDHKs and LDHa were normalized to housekeeping gene β-microglobulin.

The primers and probe sequences used for Taqman analysis were as follows:

```
PDHK1forward primer:
                                      (SEQ ID NO: 5)
GCCCATCTCATCGAAAACA

PDHK1 reverse primer:
                                      (SEQ ID NO: 6)
AGCCATCTTTAATGACTTCGACTAC

PDHK1 probe:
                                      (SEQ ID NO: 7)
TCGCAGTTTGGATTTATGCTTCCAATG

PDHK2 forward primer:
                                      (SEQ ID NO: 8)
GATCTGTCCATCAAAATGAGTGA

PDHK2 reverse primer:
                                      (SEQ ID NO: 9)
TGTGGAGTACATGTAGCTGAAGAG

PDHK2 probe:
                                     (SEQ ID NO: 10)
CTCTCAATCTTCCTCAAGGGGACACC

PDHK3 forward primer:
                                     (SEQ ID NO: 11)
CAGCCTGGAGCCTACAAGA

PDHK3 reverse primer:
                                     (SEQ ID NO: 12)
GGCATACAGTCGAGAAATTGG

PDHK3 probe:
                                     (SEQ ID NO: 13)
AAGCCATAACCAAATCCAGCCAAGG

LDHa forward primer:
                                     (SEQ ID NO: 14)
GCCGAGAGCATAATGAAGAA

LDHa reverse primer:
                                     (SEQ ID NO: 15)
CCATAGAGACCCTTAATCATGGTA

LDHa probe:
                                     (SEQ ID NO: 16)
CTTAGGCGGGTGCATCCCATTT

β-microglobulin forward primer:
                                     (SEQ ID NO: 17)
TCCTCTCAGTGGTCT GCT TGG

β-microglobulin reverse primer:
                                     (SEQ ID NO: 18)
TGGCGTGTGTAGACTTGCACTT

β-microglobulin probe:
                                     (SEQ ID NO: 19)
TGCCATCCAGCGTCCCCCA
```

Fed-Batch Shake Flask Clone Evaluation

Twelve siRNA clones and twelve mock clones were seeded into the proprietary production medium with a pH of 7.15 employing a 14-day fed-batch culture process with one bolus feed on day 3 and a temperature shift from 37° C. to 33° C. on day 2. Cell viability and viable cell counts were monitored by Trypan blue dye exclusion using a Vicell (Beckman Coulter). Lactate concentrations were measured on day 3, 7, 10 and 14 using a Nova Bioprofile analyzer (Nova biomedical). The average cell specific lactate production rate, q_S is calculated as the slope of the graph of integrated total cell number, and the cumulative lactate produced, $[S_t-S_0]$, based on the lactate mass balance equation formulated over the whole culture volume:

$$S_t - S_0 = q_s \int_0^t X\,dt$$

where S_t is the total amount of lactate in the culture volume (mg) at time t, S_0 is the total amount of lactate in the culture volume (mg) at time t=0, X is the total number of cells in the culture volume at any given time 4 and q_S is the specific lactate production rate in mg/cell/day. Since the above equation is written for the time interval between t=0 and t=t, q_S is the average lactate production rate over this time interval. Per the convention used in this work, if more lactate is produced than consumed by the cell, then the value of q_S is positive.

Bioreactor Fed-Batch Operation

Bioreactor experiments were performed in 2 L stirred tank bioreactors (Applikon, Foster City, Calif.) operated at 1.5 L working volume. After a concentrated nutrient feed at 72 hours post-inoculation, glucose was added as needed during the 14-day fed-batch culture. Dissolved oxygen and agitation were maintained in the bioreactor cultures at setpoints of 30% of air saturation and 275 rpm, respectively. Culture pH was controlled at 7.0 by addition of CO_2 gas or 1 M Na_2CO_3. Culture temperature was maintained at 37° C. for the first 48 hours, and shifted to 33° C. thereafter. Process control in each bioreactor was achieved using a Digital Control Unit from B. Braun Biotech (Allentown, Pa.).

Sample Analyses

Antibody titer was determined using conventional protein A affinity chromatography with UV detection. See Fahrner et al., Biotechnol. Appl. Biochem. 30:121-128 (1999). Culture samples were analyzed for viable cell concentration and viability by Vi-Cell AS cell counter (Beckman Coulter, Fullerton, Calif.), pH and lactate by Bioprofile 400 bioanalyzer (Nova Biomedical, Waltham, Mass.), and osmolality by a multi-sample osmometer (Advanced Instruments, Norwood, Mass.).

Statistical Analysis

Two tailed student t-test was carried out using JMP software.

Results

Construction of a siRNA Vector Targeting PDHKs and LDHa

There are four PDHK genes reported by Harris et al. (*Adv. Enzyme Regul.* 42:249-59 (2002) in mammalian cells. To assess if all four PDHK genes present in CHO cells, four sets of RT-PCR primers were designed based on the conserved regions between human and mouse PDHK sequences. The PCR results revealed that even though all four PDHK mRNAs can be detected in CHO cells, PDHK4 mRNA level is minimal and much lower than other 3 PDHKs in DHFR-deficient (dihydrofolate reductase-deficient) CHO cells. Hence, only the expression of PDHK1, 2, and 3 genes was knocked down along with LDHa gene. For LDHa and each PDHK, three siRNA sequences were designed and tested in CHO cells to choose the siRNA sequence exhibiting best down-regulation of the target gene. The best siRNA sequence for LDHa was selected based on the findings by Kim and Lee. *Appl. Microbiol. Biotechnol.* 74(1):152-9 (2007). The siRNA sequence for LDHa and PDHKs were constructed in a single vector where siRNA for LDHa was under the control of U6 promoter, whereas siRNAs for each PDHK were driven by H1 promoters (FIG. **1**).

Generation of Stable Clones with Reduced Expression of PDHK1, 2, 3, and LDHa

The siRNA construct targeting PDHKs and LDHa was transfected into CHO cells expressing a monoclonal antibody to get individual clones named siRNA clones. Individual siRNA clones were assayed for mRNA expression of four genes, PDHK1, 2, 3 and LDHa, using Taqman analysis. Twelve siRNA clones that exhibited most reduced expression of above four genes were identified (FIG. **2**) for further analysis. The mock vector containing scramble sequence was also transfected into the same antibody expressing cells to get individual clones named mock clones. Twelve mock clones were chosen randomly as control and their mRNA expression levels of LDHa and PDHK1, 2, and 3 genes were also analyzed by Taqman. On average, the mRNA expression levels for LDHa, PDHK1, 2, and 3 in selected twelve siRNA clones were reduced by 90%, 32%, 83%, and 70% respectively compared to mock clones (FIG. **2**).

Fed-Batch Shake Flask Evaluation of siRNA and Mock Clones

(a) Reduced Lactate Levels and Higher pHs in Culture Media Observed in siRNA Clones

To evaluate the effect of siRNA-mediated down-regulation of LDHa and PDHKs on lactate production, 12 siRNA and 12 mock clones were evaluated in shake flask vessels in our proprietary medium employing a 14-day, fed-batch, and temperature shift process. The experiment has been repeated for three times and similar results were observed. The results from one set of experiment is shown as representatives in the figures. The results showed that compared to mock clones, siRNA clones had reduced lactate levels (FIG. **3**) in general. By day 14, siRNA clones showed 91% less lactate in average than mock clones ($p<0.0001$) (FIG. **3**A). Consistent with the lower lactate level in siRNA clones over the 14-day production period, the average lactate production rate for siRNA clones was negative 0.02 mg/10^6 cells/day, suggesting that lactate synthesis rate is lower than the consumption rate. In contrast, the average lactate production rate was 0.01 mg/10^6 cells/day for mock clones, indicating the overall lactate synthesis rate is higher than the consumption rate. This difference in lactate production rate between siRNA and mock clones was statistically significant ($p<0.002$) (FIG. **3**B). Since lactate level in the media affects pH, by day 14, the average pH for mock clones dropped to 6.54, whereas the average pH for siRNA clones was 7.04 (FIG. **3**C). The observed lower average pH is in agreement with higher average lactate level for mock clones.

b) Increased Antibody Titer and Specific Productivity (Qp) Observed in siRNA Clones

To investigate whether knocking down gene expression of PDHKs and LDHa affect antibody production, samples were collected from fed-batch shake flask experiments on day 3, 7, 10 and 14 to measure antibody titers by protein A chromatography. The data showed that, on average, siRNA clones produced 68% more antibody that that of mock clones (FIG. **4**A, $p<0.022$), and average cell-specific productivity (Qp) measured in pg/cell-d for siRNA clones was 75% higher than that for mock clones (FIG. **4**B, $p<0.006$). To evaluate cell growth, shake flask samples were collected on day 3, 7, 10, and 14 to measure viable cell counts and viabilities to calculate integrated viable cell count (IVCC). In contrast to antibody titers and Qps, no appreciable cell growth differences were observed between the two groups (FIG. **4**C). Antibody product quality attributes including glycan profile, charge variants and percentage of aggregation were comparable between siRNA and mock clones.

Bioreactor Fed-Batch Culture Evaluation of siRNA Mock Clones

Since pH-controlled fed-batch bioreactor culture is the standard scale-down model for large scale manufacturing, the performance of some siRNA and mock clones in 2 L bioreactors was further investigated. Given the limitation in bioreactor availability and experimental complexity, 12 siRNA and 12 mock clones in duplicates were not run due to impracticability. Two representative siRNA clones and two representative mock clones whose metabolic profiles best represented the average performance for each group to minimize selection bias, along with the parental line used for siRNA and mock plasmid transfections for 2 L bioreactor evaluation were selected. Cell culture samples were collected daily (except on days 6 and 13) for lactate, glucose, osmolality, and titer analysis. The lactate levels for siRNA clones generally remained flat whereas the lactate levels for mock and parental clones continued to increase during the 14-day production period. On day 14, the two siRNA clones had 86% lower lactate level on average in media than mock clones or parental clone (FIG. **5**A) and had lower specific lactate production rate than mock clones and parent line (FIG. **5**B). Similarly, the osmolarities for siRNA clones remained around 300 mOsm whereas the osmolarities for mock clones or parental clone continued to increase during the 14-day production period. On day 14, average osmolarities for 2 siRNA clones were 60% lower than those of mock and parent clones (FIG. **5**C). Importantly, on day 14, the siRNA clones on average produced 125% more antibody than that of mock clones (FIG. **6**). As observed in fed-batch shake flask evaluation, siRNA and mock clones have comparable viabilities and cell growth in 2 L bioreactors.

Discussion

Previous study demonstrated that down-regulating LDHa gene expression alone was able to reduce lactate production. Kim and Lee, *Appl. Microbiol. Biotechnol.* 74(1):152-9 (2007). However, in their study despite the 45-79% reduction in lactate level, there was no significant improvement in Qp and product titer suggesting that knocking down LDHa alone in CHO cells is not sufficient to improve Qp and product yield efficiently. Further, simultaneously down-regulating PDHK1, 2, and 3 in CHO cells was neither sufficient to reduce lactate level nor to increase antibody productivity. Since the only way for cells to generate lactate is through pyruvate reduction, and pyruvate can not only be converted to lactate by LDH but also be converted to acetyl-CoA by PDH entering TCA cycle to be oxidized, reducing lactate production by knocking down LDHa expression and promoting pyruvate into TCA cycle by knocking down PDHKs may synergize to reduce lactate level and to provide cells with more energy and possibly metabolic intermediates leading to increased antibody production.

The expression of LDHa, PDHK2, and PDHK3 was substantially reduced and the expression of PDHK1 was moderately reduced in all clones tested. The moderate reduction in PDHK1 expression is likely due to non-optimum siRNA targeting sequence since moderate reduction was observed with three PDHK1 siRNA sequences tested. Variations on lactate production and antibody production in mock and siRNA clones were observed, since each clone had different expression levels of LDHa and PDHKs. Nevertheless, by day 14, the average lactate level in siRNA group was lower than that in mock group leading to the lower average pH for mock clones than that of siRNA clones in fed-batch shake flask culture. More importantly, in addition to lower specific lactate production rate, the average

titer and Qp for siRNA clones increased by 68% and 75% respectively compared to those of mock clones with no noticeable differences in cell growth and product quality between siRNA and mock clones. Interestingly, for the day 14 titers versus day 14 lactate levels, there was a good inverse relationship between titers and lactate levels among mock clones, but not among siRNA clones. The observed differences in titers and lactate levels among mock clones may be likely that parental clone is heterogeneous in antibody productivity and cellular metabolism even though the cell line was derived from a single clone. A total of 12 mock clones were evaluated to take into consideration of clonal variation. The data indicate that knocking down LDHa and PDHKs simultaneously reduces lactate level and improves antibody production in CHO cells. Hence, for the development of robust and productive antibody production processes, simultaneous down regulation of both LDHa and PDHKs provides an efficient approach.

The performance of 2 mock and 2 siRNA clones in 2 L bioreactors with duplicates was further investigated. Those 4 clones were selected to best represent the average productivity in each group based on fed-batch shake flask evaluations. Similar to the observations from shake flask experiment, the siRNA clones had lower lactate levels and higher titers than mock clones in 2 L bioreactor evaluation. Given that pH is controlled in fed-batch 2 L bioreactors, the mock cultures exhibited increased osmolality than siRNA cultures since higher lactate levels in mock clones needed more alkali addition to maintain set point pH.

In summary, the data from fed-batch shake flask and 2 L bioreactor evaluations demonstrated that simultaneous knockdown of LDHa, PDHK1, 2, and 3 in CHO cells is effective in reducing lactate level and in increasing antibody titer without impacting cell growth and product quality.

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What is claimed is:

1. A method for reducing lactate production in cultured cells, the method comprising culturing cells comprising a first heterologous nucleic acid sequence encoding a small interfering RNA (siRNA) specific for a lactate dehydrogenase (LDH) and a second heterologous nucleic acid sequence encoding an siRNA specific for a pyruvate dehydrogenase kinase (PDHK), wherein the first heterologous nucleic acid sequence is operably linked to a first promoter, and wherein the second heterologous nucleic acid sequence is operably linked to a second promoter, wherein the cultured cells have a polypeptide productivity of at least about 68% higher than cultured cells without the heterologous nucleic acid sequence comprising siRNA specific for PDHK and the siRNA specific for LDH.

2. The method of claim **1**, wherein the LDH is LDHa.

3. The method of claim **1**, wherein the cultured cells further comprise a third heterologous nucleic acid sequence encoding an siRNA specific for a second PDHK and wherein the third heterologous nucleic acid sequence is operably linked to a third promoter.

4. The method of claim **3**, wherein the cultured cells further comprise a fourth heterologous nucleic acid sequence encoding an siRNA specific for a third PDHK and wherein the fourth heterologous nucleic acid sequence is operably linked to a fourth promoter.

5. The method of claim **4**, wherein cultured cells further comprise a fifth heterologous nucleic acid sequence encoding an siRNA specific for a fourth PDHK and wherein the fifth heterologous nucleic acid sequence is operably linked to a fifth promoter.

6. The method of any one of claims **1**, **3**, **4**, and **5**, wherein the PDHK is selected from the group consisting of PDHK1, PDHK2, PDHK3, and PDHK4.

7. The method of claim **1**, wherein the cultured cells produce a heterologous polypeptide.

8. The method of claim **7**, wherein the heterologous polypeptide is an antibody.

9. The method of claim **4**, wherein an average lactate production rate of the cultured cells is less than about negative 0.02 mg/10^6 cells/day.

10. The method of claim **4**, wherein the cultured cells have a Specific Productivity of at least about 75% higher than cultured cells without the heterologous nucleic acid sequence comprising the siRNA specific for PDHKs and the siRNA specific for LDH.

11. The method of claim **4**, wherein the cultured cells maintain an osmolality at less than about 300 mOsm over 14 days.

12. The method of claim **7**, wherein the cultured cells are mammalian cells.

* * * * *

EXHIBIT HH

US009714293B2

(12) **United States Patent**
Gawlitzek et al.

(10) **Patent No.: US 9,714,293 B2**
(45) **Date of Patent: *Jul. 25, 2017**

(54) **PRODUCTION OF PROTEINS IN GLUTAMINE-FREE CELL CULTURE MEDIA**

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(72) Inventors: **Martin Gawlitzek**, Redwood City, CA (US); **Shun Luo**, Irvine, CA (US); **Christina Teresa Bevilacqua**, San Ramon, CA (US)

(73) Assignee: **GENENTECH, INC.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **14/670,079**

(22) Filed: **Mar. 26, 2015**

(65) **Prior Publication Data**

US 2016/0024217 A1 Jan. 28, 2016

Related U.S. Application Data

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(60) Provisional application No. 61/232,889, filed on Aug. 11, 2009.

(51) **Int. Cl.**

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C07K 16/28	(2006.01)
C12N 5/00	(2006.01)
C07K 16/18	(2006.01)
C07K 16/22	(2006.01)
C07K 14/705	(2006.01)

(52) **U.S. Cl.**

CPC ***C07K 16/2878*** (2013.01); ***C07K 14/70575*** (2013.01); ***C07K 16/18*** (2013.01); ***C07K 16/22*** (2013.01); ***C12N 5/0018*** (2013.01); ***C12N 5/0043*** (2013.01); *C07K 2317/14* (2013.01); *C07K 2319/30* (2013.01); *C12N 2500/32* (2013.01); *C12N 2500/33* (2013.01); *C12N 2500/90* (2013.01)

(58) **Field of Classification Search**

CPC C12N 5/18

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,049,494 A	*	9/1977	Tomei	A61K 39/135 424/216.1
4,399,216 A		8/1983	Axel et al.	
4,515,893 A		5/1985	Kung et al.	
4,560,655 A		12/1985	Baker et al.	
4,657,866 A		4/1987	Kumar	
4,766,075 A		8/1988	Goeddel et al.	
4,767,704 A		8/1988	Cleveland et al.	
4,816,567 A		3/1989	Cabilly et al.	
4,853,330 A		8/1989	Goeddel et al.	
4,927,762 A		5/1990	Darfler	
5,091,313 A		2/1992	Chang	
5,122,469 A		6/1992	Mather et al.	
5,185,259 A		2/1993	Goeddel et al.	
5,500,362 A		3/1996	Robinson et al.	
5,545,806 A		8/1996	Lonberg et al.	
5,545,807 A		8/1996	Surani et al.	
5,569,825 A		10/1996	Lonberg et al.	
5,622,700 A		4/1997	Jardieu et al.	
5,625,126 A		4/1997	Longberg et al.	
5,633,425 A		5/1997	Lonberg et al.	
5,641,870 A		6/1997	Rinderknecht et al.	
5,661,016 A		8/1997	Lonberg et al.	
5,672,347 A		9/1997	Aggarwal et al.	
5,693,762 A		12/1997	Queen et al.	
5,714,338 A		2/1998	Wai Fei et al.	
5,721,108 A		2/1998	Robinson et al.	
5,725,856 A		3/1998	Hudziak et al.	
5,736,137 A		4/1998	Anderson et al.	
5,750,373 A		5/1998	Garrad et al.	
5,776,456 A		7/1998	Anderson et al.	
5,821,333 A		10/1998	Carter et al.	
5,821,337 A		10/1998	Carter et al.	
5,843,439 A		12/1998	Anderson et al.	
6,075,181 A		6/2000	Kucherlapati et al.	
6,150,584 A		11/2000	Kucherlapati et al.	
6,171,586 B1		1/2001	Lam et al.	
6,194,551 B1		2/2001	Idusogie et al.	

(Continued)

FOREIGN PATENT DOCUMENTS

EP	0117058 A2	8/1984
EP	0117059 A2	8/1984

(Continued)

OTHER PUBLICATIONS

Nagle et al. 1971; An improved heat-stable glutamine-free chemically defined medium for growth of mammalian cells. J. Cell Physiol. 77: 259-264.*
Kurano et al. 1990; Growth behavior of Chinese hamster ovary cells in a compact loop bioreactor. 2. Effects of medium components and waste products. J. Biotechnol. 15: 113-128.*
products.invitrogen.com/ivgn/product/A1049001, 2012.*
Sanfeliu et al. (1999; Effect of glutamine limitation on the death of attached Chinese hamster ovary cells. Biotechnol. Bioeng. 64: 46-53.*
sigmaaldrich.com/life-science/cell-culture/learning-center/media-formulations, 2012.*
de la Cruz Edmonds et al. 2006; Development of transfection and high-producer screening protocols for the CHOK1SV cell system. Molecular Biotechnology 34: 179-190.*

(Continued)

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(74) *Attorney, Agent, or Firm* — Morrison & Foerster LLP

(57) **ABSTRACT**

The present invention relates generally to glutamine-free cell culture media supplemented with asparagine. The invention further concerns the production of recombinant proteins, such as antibodies, in asparagine-supplemented glutamine-free mammalian cell culture.

78 Claims, 25 Drawing Sheets

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,242,195 B1 6/2001 Idusogie et al.
6,399,061 B1 6/2002 Anderson et al.
6,455,043 B1 9/2002 Grillo-Lopez
6,528,624 B1 3/2003 Idusogie et al.
6,538,124 B1 3/2003 Idusogie et al.
6,610,516 B1 8/2003 Anderson et al.
6,982,321 B2 1/2006 Winter
7,060,269 B1 6/2006 Baca et al.
7,087,409 B2 8/2006 Barbas, III et al.
7,169,901 B2 1/2007 Baca et al.
7,297,334 B2 11/2007 Baca et al.
8,512,983 B2 8/2013 Gawlitzek et al.
2002/0004587 A1 1/2002 Miller et al.
2002/0006404 A1 1/2002 Hanna
2002/0009444 A1 1/2002 Grillo-Lopez
2002/0012665 A1 1/2002 Hanna
2002/0058029 A1 5/2002 Hanna
2002/0128488 A1 9/2002 Yamakawa et al.
2002/0197255 A1 12/2002 Andeson et al.
2002/0197256 A1 12/2002 Grewal
2003/0021781 A1 1/2003 Anderson et al.
2003/0082172 A1 5/2003 Anderson et al.
2003/0095963 A1 5/2003 Anderson et al.
2003/0103971 A1 6/2003 Hariharan et al.
2003/0147885 A1 8/2003 Anderson et al.
2004/0186051 A1 9/2004 Kelley et al.
2005/0070689 A1* 3/2005 Dixit C07K 16/2878
530/350
2006/0246004 A1 11/2006 Adams et al.
2011/0091936 A1 4/2011 Gawlitzek et al.
2014/0024078 A1 1/2014 Gawlitzek et al.

FOREIGN PATENT DOCUMENTS

EP 0117060 A2 8/1984
EP 0307247 A2 3/1989
EP 0404097 A2 12/1990
EP 0420937 A1 4/1991
EP 2464725 A1 6/2012
FR WO 2005083058 A1 * 9/2005 .... C07K 14/47
GB 2237288 A 5/1991
WO WO-87/00195 A1 1/1987
WO WO-89/05859 A1 8/1989
WO WO-90/03430 A1 4/1990
WO WO-91/00358 A1 1/1991
WO WO-91/10741 A1 7/1991
WO WO-93/04173 A1 3/1993
WO WO-93/11161 A1 6/1993
WO WO-95/19181 A1 7/1995
WO WO-95/23865 A1 9/1995
WO WO-96/30046 A1 10/1996
WO WO-96/30500 A1 10/1996
WO WO-96/33735 A1 10/1996
WO WO-96/34096 A1 10/1996
WO WO-96/40210 A1 12/1996
WO WO-97/25428 A1 7/1997
WO WO-97/26912 A2 7/1997
WO WO-98/06248 A2 2/1998
WO WO-98/23761 A1 6/1998
WO WO-98/24893 A2 6/1998
WO WO-98/45331 A2 10/1998
WO WO-98/51793 A1 11/1998
WO WO-98/56418 A1 12/1998
WO WO-98/58964 A1 12/1998
WO WO-99/22764 A1 5/1999
WO WO-99/51642 A1 10/1999
WO WO-00/09160 A1 2/2000
WO WO-00/27428 A1 5/2000
WO WO-00/27433 A1 5/2000
WO WO-00/42072 A2 7/2000
WO WO-00/44788 A1 8/2000
WO WO-00/67796 A1 11/2000
WO WO-00/75348 A1 12/2000
WO WO-01/03734 A1 1/2001
WO WO-01/10460 A1 2/2001
WO WO-01/10461 A1 2/2001
WO WO-01/10462 A1 2/2001
WO WO-01/40309 A2 6/2001
WO WO-01/74388 A1 10/2001
WO WO-01/77342 A1 10/2001
WO WO-01/80884 A1 11/2001
WO WO-01/97858 A2 12/2001
WO WO-02/04021 A1 1/2002
WO WO-02/34790 A1 5/2002
WO WO-02/060955 A2 8/2002
WO WO-02/079255 A1 10/2002
WO WO-02/096948 A2 12/2002
WO WO-2004/092219 A2 10/2004
WO WO 2006083971 A2 * 8/2006 .... C07K 16/2878
WO WO-2008/008482 A2 1/2008

OTHER PUBLICATIONS

Invitrogen.com/1/1/10163-cd-cho-medium-1x-liquid.html, 2012.*
Wu et al. 2001; Multimerization of chimeric anti-CD20 single-chain Fv-Fc fusion protein is mediated through variable domain exchange. Protein Engineering 14(12): 1025-1033.*
Beck et al. 2008; Trends in glycosylation, glycoanalysis, and glycoengineering of therapeutic antibodies and Fc-Fusion proteins. Current Pharmaceutical Biotechnology 9(6): 482-501.*
Dyring et al. 1994; Observations on the influence of glutamine, asparagine, and peptone on growth and t-PA production of Chinese hamster ovary (DHO) cells. Cytotechnology 16:37-42.*
Altamirano, et al., "Strategies for fed-batch cultivation oft-PA producing CHO cells: substitution of glucose and glutamine and rational design of culture medium", Journal of Biotechnology, 110: 171-179, (2004).
Aruffo et al. "CD44 Is the Principal Cell Surface Receptor for Hyaluronate", Cell vol. 61, 1303-1313, 1990.
Barbas et al. "In Vitro Evolution of a Neutralizing Human Antibody to Human Immunodeficiency Virus Type 1 to Enhance Affinity and Broaden Strain Cross-Reactivity", Proc. Natl. Acad. Sci.USA; vol. 91, pp. 3809-3813, 1994.
Barnes and Sato "Methods for Growth of Cultured Cells in Serum-Free Medium", Analytical Biochemistry 102: 255, 1980.
Barnes et al. "Serum-Free Cell Culture: a Unifying Approach", Cell, vol. 22, 649-655,1980.
Baselga et al., Phase II Study of Weekly Intravenous Recombi11llill: Hum311ized Anti-pl 85HER2 Monoclonal Antibody in Patients with HER2/neu-Overexpressing Metastatic Breast Cancer, vol. 14, No. 3, pp. 737-744, Mar. 1996, Journal of Clinical Oncology, American Society of Clinical Oncology.
Baumann et al. "Dexammethasone Regulates the Program of Secretory Glycoprotein Synthesis in Hepatoma Tissue Culture Cells", J. Cell Biology, vol. 85, pp. 1-8, 1980.
Bird et al. "Single-Chain Antigen-Binding proteins", Science vol. 242, pp. 423-426, 1988.
Boerner al "Production of antigen-specific human monoclonal antibodies from in vitro-primed human splenocytes:", J. ofImmunology, vol. 147, No. 1, pp. 86-95, 1991.
Brennan et al. "Preparation of Bispecific Antibodies by Chemical Recombination of Monoclonal Immunoglobulin G1 Fragments", Science 229:81, 1985.
Brodeur et al., Monoclonal Antibody Production Techniques and Applications, pp. 51-63, 1987 (Marcel Dekker, Inc., New York).
Bruggemann et al. "Designer Mice: The Production of Human Antibody Repertoires in Transgenic Animals", Year Immunol. vol. 7, p. 33-40, 1993.
Burton, D.R. "Immunoglobulin G:Functional Sites", Molecular Immunology vol. 22:161-206, 1985.
Capel et al. "Heterogeneity ofhuman IgG Fe receptors", Immunomethods vol. 4, pp. 25-34,1994.
Carter et al. "High Level *Escherichia coli* Expression and Production of a Bivalent Humanized Antibody Fragment", Biotechnology 10:163-167, 1992.
Clackson et al. "Making antibody fragments using phage display libraries", Nature, vol. 352, pp. 624-628, 1991.

(56) **References Cited**

OTHER PUBLICATIONS

Clynes et al. "Fe receptors are required in passive and active immunity to melanoma", Proc. Natl. Acad. Sci. (USA); vol. 95, pp. 652-656, 1998.
Cole et al. "The EBV-Hybridoma Technique and its application to Human Lung Cancer" Monoclonal Antibodies and Cancer Therapy, pp. 77-96, 1985.
Daeron M. "Fe Receptor Biology", Annu. Rev. Immunol.: 15: 203-34, 1997.
Duchosal et al. "Immunization ofhu-PBL-SCID Mice and the Rescue of Human Monoclonal Fab Fragments through Combinatorial Libraries" Nature 355:258, 1992.
Fellouse et al. "Synthetic Antibodies from a Four-Amino-Acid Code: A Dominant role for Tyrosine in Antigen Recognition", PNAS: vol. 101; No. 34: 12467-12472, 2004.
Fishwild et al "High-Avidity Human IgGk Monoclonal Antibodies from a Novel Strain of Minilocus Transgenic Mice", Nature Biotech; vol. 14, pp. 845-851, 1996.
Gasser et al. "Expression of Abbreviated Mouse Dihydrofolate Reductase Genes in Cultured Hamster Cells", Proc. Natl. Acad. Sci. USA vol. 79, pp. 6522-6526, 1982.
Gazzano-Santoro et al. "A Non-Radioactive Complement-Dependent Cytotoxicity Assay for Anti-CD20 Monoclonal Antibody", J ofImmunol Methods 202, pp. 163-171, 1997.
Gething et al. "Cell-surface Expression ofInfluenza Haemagglutinin from a Cloned DNA copy of the RNA Gene", Nature vol. 293: 620-625, 1981.
Ghetie et al. "FcRn: the MHC Class I-Related Receptor that is more than an IgG Transporter" vol. 18, No. 12, pp. 592-598, 1997.
Ghetie et al. "Increasing the Serum Persistence of an IgG Fragment by Random Mutagenesis", Nature Biotech vol. 15, pp. 637-640, 1997.
Goding J.W. "Monoclonal Antibodies: Principles and Practice" nn.59-103 (Academic Press, 1986).
Graham et al. "Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5", J. Gen. Virol: 36, 59-72, 1977.
Graham et al. "A New Technique for the Assay ofInfectivity of Human Adenovirus 5 DNA" Virology 52, 456-467, 1973.
Gruber et al. "Efficient Tumor Cell Lysis Mediated by a Bispecific Single Chain Antibody Expressed in *Escherichia coli*" J. Immunol. 152:5368, 1994.
Gueffroy, D. "A Guide for the Preparation and Use of buffers in Biological Systems" Cal Biochem Corporation, 1975.
Guyer et al. "Immunoglobulin Binding by Mouse Intestinal Epithelial Cell Receptors" Journal of Immunology; vol. 117, No. 2, pp. 587-593, 1976.
Haas et al. "FcY receptors of phagocytes", J. Lab. Clin Med 126: 330-41, 1995.
Ham et al. "Media and Growth Requirements" Meth. Enz., 58: 44, 1979.
Hamers-Casterman et al. "Naturally Occurring Antibodies Devoid of Light Chains", Nature vol. 363, pp. 446-448, 1993.
Hammerling et al. "Monoclonal Antibodies and T-Cell Hybridomas", Elsevier; pp. 563-681, 1981.
Harris et al. "Production of Humanized Monoclonal Antibodies for in Vivo Imaging and Therapy", Biochemical Society Transactions; vol. 23, pp. 1035-1038, 1995.
Hawkins et al. "Selection of Phage Antibodies by Binding Affinity Mimicking Affinity Maturation", J. Mol. Biol.; vol. 226, pp. 889-896, 1992.
Hinton et al. "Engineered Human IgG Antibodies with Longer Serum Half-lives in Primates", J. Biol. Chemistry, vol. 279, No. 8, pp. 6213-6216, 2004.
Holliger et al. "Diabodies: Small Bivalent and Bispecific Antibody Fragments" Proc. Natl. Acad. Sci. USA; vol. 90, pp. 6444-6448, 1993.
Hongo et al. Development and Characterization ofMurine Monoclonal Antibodies to the Latency-Associated Peptide of Transforming Growth Factor ~I ; Hybridoma; vol. 14, No. 3, pp. 253-260, 1995.
Hoogenboom et al. "By-passing Immunisation Human Antibodies from Synthetic Repertoires of Germline Vh Gene Segments Rearranged in Vitro", J. Mol. Biol. vol. 227, pp. 381-388, 1992.
Hoogenboom et al. "Construction and Expression of Antibody-Tumor Necrosis Factor Fusion Proteins", Mol. Immunol. vol. 28, No. 9, pp. 1027-1037, 1991.
Huang et al. "Nitrogen metabolism of asparagine and glutamate in Vero cells studied by 1H/15N NMR spectroscopy", Applied Microbiology and Biotechnology, Springer, Berlin, DE LNKD—DOI: 10.1007/S00253-007-I 181-8, vol. 77, No. 2, Oct. 19, 2007 (Oct. 19, 2007), pp. 427-436, XP01956071 I, ISNN: 1432-0614.
Hudson et al. "Engineered Antibodies" Nature Med. vol. 9, pp. 129-134, 2003.
Hurle et al. "Protein Engineering Techniques for Antibody Humanization", Current Opinion Biotech.: 5: 428-433, 1994.
Huston et al. "Protein Engineering of Antibody Binding Sites: Recovery of Specific Activity in an Anti-Digoxin Single-Chain Fv Analogue Produced in *Escherichia! coli*", PNAS (USA); vol. 85, pp. 5879-5883, 1988.
Idusogie et al. "Mapping of the Clq Binding Site on Rituxan, a Chimeric Antibody with a Human IgGI Fe", J. Immunol. vol. 164: 4178-4184, 2000.
Jackson et al. "In Vitro Antibody Maturation", J. Immunol: 154:3310-3319, 1995.
Jakobovits et al. "Analysis of Homozygous Mutant Chimeric Mice: Deletion of the Immunoglobulin Heavy-Chain Joining Region Blocks B-cell Development and Antibody Production" Proc. Natl. Acad. Sci. USA; vol. 90, pp. 2551-2555, 1993.
Jakobovits et al. "Germ-line Transmission and Expression of a Human-derived Yeast Artificial Chromosome", Nature, vol. 362, pp. 255-258, 1993.
Johnson et al. "The Kabat Database and a Bioinformatics Example", Methods in Molecular Biology; vol. 248, pp. 11-25, 2003.
Jones et al. "Replacing the Complementarity-Determining Regions in a Human Antibody with those from a mouse" Nature, vol. 321, pp. 522-525, 1986.
Kaufman et al. "Amplification and Expression of Sequences Cotransfected with a Modular Dihydrofolate Reductase Complementary DNA Gene", J. Mol. Biol. 159: 601-621, 1982.
Keown et al. "Methods for Introducing DNA into Mammalian Cells", Methods in Enzymology: 185:527-537, 1990.
Kohler et al. "Continuous Cultures of Fused Cells Secreting Antibody of Predefined Specificity", Nature, vol. 256, pp. 495-497, 1975.
Kostelny et al. "Formation of a Bispecific Antibody by the Use ofLeucine Zippers" J. Immunol. 148(5):1547-1553, 1992.
Kozbor et al. "A Human Hybrid Myeloma for Production of Human Monoclonal Antibodies", Journal Immunology 133:3001, 1984.
Lee et al. "High-affinity Human Antibodies from Phage-displayed Synthetic Fab Libraries with a Single Framework Scaffold", J. Mol. Biol. 340, 1073-1093, 2004.
Lee et al. "Bivalent Antibody Phage Display Mimics Natural Immunoglobulin", J Immunoligical Methods :284, pp. 119-132, 2004.
Li et al. "Human Antibodies for Immunotherapy Development Generated via a Human B Cell Hybridoma Technology", PNAS (USA) vol. 103, pp. 3557-3562, 2006.
Lonberg et al. "Antigen-Specific Human Antibodies from Mice Comprising Four Distinct Genetic Modifications", Nature: vol. 368, pp. 856-859, 1994.
Lonberg et al. "Human Antibodies from Transgenic Mice" , Intern. Rev. Immunol. vol. 13, pp. 65-93, 1995.
Lubiniecki et al. "Advances in Animal Cell Biology and Technology for Bioprocesses", ESACT pp. 442-451, 1989.
Luckow et al. "Trends in the Development o fBaculovirus Expression Vectors", Biotechnology, vol. 6, pp. 47-55, 1988.
Maeda et al. "Production of Human a-interferon in Silkworm Using a Baculovirus Vector", Nature, vol. 315, pp. 592-594, 1985.

(56) **References Cited**

OTHER PUBLICATIONS

Maloney et al., IDEC-C2B8 (Ritl.Lximab) Anti-CD20 Monoclonal Antibody Therapy in Patients With Relapsed Low-Grade Non-Hodgkm's Lymphoma, vol. 90, No. 6. pp. 2188-2195, Sep. 1997, Blood, American Society of Hematology, Washington, D.C.
Mantei et al. "Rabbit ~-globin Mma Production in Mouse L Cells Transformed with Cloned Rabbit ~-globin Chromosomal DNA", Nature, 281 :40-46, 1979.
Mansour et al. "Disruption of the Proto-Oncogene *int-2* in Mouse Embryo-Derived Stem Cells: a General Strategy for Targeting Mutations to Non-selectable Genes", Nature, 336:348-352, 1988.
Marks et al. "By-passing Immunization Human Antibodies from V-gene Libraries Displayed on Phage" J. Mol. Biol. vol. 222, pp. 581-597, 1991.
Marks et al. "By-passing Immunization: Building High Affinity Human Antibodies by Chain Shuffling" Biotechnology; vol. 10, pp. 779-783, 1992.
Mather, J.P. "Establishment and Characterization of Two Distinct Mouse Testicular Epithelial Cell Lines" Biology of Reproduction 23, p. 243-252 ,1980.
Mather et al. "Culture of Testicular Cells in Hormone-Supplemented Serum-Free Medium", NY AS ; vol. 0383-0044, p. 44, 1982.
Maxam et al. "Sequencing End-Labeled DNA with Base-Specific Chemical Cleavages", Methods in Enzymology, 65:499, 1980.
McCafferty et al. "Phage Antibodies: Filamentous Phage Displaying Antibody Variable Domains", Nature, 348:552-554, 1990.
Messing et al. "A System for Shotgun DNA Sequencing", Nuceleic Acids Research 9:309, 1981.
Miller et al. Genetic. Engineering Setlow; Plenum Publishing; vol. 8, pp. 277-279, 1986.
Miller et al. "A kinetic analysis ofhybridoma growth and metabolism in batch and continuous suspension culture: Effect of nutrient concentration, dilution rate, and pH", Biotechnology and Bioengineering, vol. 32, pp. 947-965, (1988).
Millstein et al. "Hybrid Hybridomas and their use in Immunohistochemistry", Nature vol. 305:537-539, 1983.
Morimoto et al. Single-step Purification ofF(ab')2 Journal of Biochemical and Biophysical Methods 24:107-117,1992.
Morrison, S.L. "Success in Specification" Nature, vol. 368, pp. 812-813, 1994.
Morrison et al. "Chimeric Human Antibody Molecules: Mouse Antigen-binding Domains with Human Constant Region Domains", Proc. Natl. Acad. Sci.USA, vol. 81, pp. 6851-6855,1984.
Neuberger, M. "Generating High-Avidity Human Mabs in Mice", Nature, vol. 14, p. 826, 1996.
Pluckthun, A. "The Pharmacology of Monoclonal Antibodies", Springer-Verlag vol. 113, pp. 269-315, 1994.
Presta, L.G. "Antibody Engineering" Current Opinion in Struct Biol. 2:593-596, 1992.
Presta et al. "Humanization of an Antibody Directed Against IgE", J. Immnol., 151 :2623-2632, 1993.
Presta et al., Humanization of an Anti-Vascular Endothelial Growth Factor Monoclonal Antibody for the Therapy of Solid Tumors and Other Disorders, vol. 57, pp. 4593-4599, Oct. 1997, Cancer Research, American Association for Cancer Research.
Ravetch et al. "Fe Receptors", Annu. Rev. Immunol. 9: 457-92, 1991.
Riechmann et al. "Reshaping Human Antibodies for Therapy", Nature, vol. 332, pp. 323-329, 1988.
Schier et al. "Identification of Functional and Structural Amino-Acid Residues by Parsimonious Mutagenesis", Gene, 169, pp. 147-155, 1996.
Schneider et al., "The importance of ammonia in mammalian cell culture", Journal of Biotechnology, 46: 161-185, (1996).
Shalaby et al. "Development of Humanized Bispecific Antibodies Reactive with Cytotoxic Lymphocytes and Tumor Cells Overexpressing the HER2 Protooncogene", J. Exp. Med. 175:217-225,1992.
Shaw et al. "A General Method for the Transfer of Cloned Genes to Plant Cells", Gene 23:315, 1983.
Sheets et al. "Efficient Construction of a large Nonimmune Phage Antibody Library: The Production of High-Affinity Human Single-Chain Antibodies to Protein Antigens", PNAS (USA) vol. 95, pp. 6157-6162, 1998.
Sheriff et al. "Redefining the Minimal Antigen-Binding Frgament", Nature Structural Biology vol. 3, pp. 733-736, 1996.
Shields et al. "High Resolution Mapping of the Binding Site on Human IgG 1 for FCYRI, FCYRII, FCYRIII, and FcRn and Design ofIgG 1 Variants with Improved Binding to the FCYR"; J. Biol. Chem. vol. 276, No. 9, pp. 6591-6604, 2001.
Sidhu et al. "Phage-displayed antibody libraries of synthetic heavy chain complementarity determining regions" J. Mol.. Biol. vol. 338, No. 2, pp. 299-310, 2004.
Simonsen et al. "Isolation and expression of an altered mouse dihydrofolate reductase cDNA" Proc. Natl. Acad. Sci. USA, vol. 80, pp. 249-2499, 1983.
Sims et al. "A Humanized CD 18 Antibody Can Block Function without Cell Destruction", Journal Immunol., 151 :2296 , 1993.
Stamenkovic et al. "The B Lymphocyte Adhesion Molecule CD22 Interacts with Leukocyte Common Antigen CD45RO on T Cells and a2-6 Sialyltransferase, CD75, on B Cells" Cell vol. 66, 1133-1144, 1991.
Thomas, P. "Hybridization of Denatured RNA and small DNA Fragments Transferred to Nitrocellulose", Proc. Natl. Acad. Sci. USA: 77:5201-5205, 1980.
Traunecker et al. "Bispecific Single Chain Molecules (Janusins) target Cytotoxic Lymphocytes on HIV Infected Cells", EMBO Journal vol. 10 No. 12, pp. 3655-3659, 1991.
Trill et al. "Production of Monoclonal Antibodies in COS and CHO cells", Current Opinion Biotech 6:553-560, 1995.
Tutt et al. "Trispecific F(ab')3 Derivatives that Use Cooperative Signaling via the TCR/CD3 Complex and CD2 to Activate and Redirect Resting Cytotoxic T Cells 1" J. Immunol. 147:60-69, 1991.
Urlaub et al. "Isolation of Chinese Hamster Cell Mutants Deficient in Dihydrofolate Reductase Activity" Proc. Natl. Acad. Sci. USA vol. 77, No. 7, p. 4216-4220, 1980.
Van Dijk et al. "Human Antibodies as Next Generation Therapeutics" Current Opinion in Chem. Biol. 5:368-374, 2001.
Vaswani et al. "Humanized antibodies as potential therapeutic drugs", Ann Allergy Asthma Immunol. 81: 105-119, 1998.
Vaughan et al. "Human antibodies with sub-nanomolar affinities isolated from a large nonimmunized phage display library", Nature Biotechnolo!!v, vol. 14, pp. 309-314, 1996.
Verhoeyen et al. "Reshaping Human Antibodies: Grafting an Antilysozyme Activity", Science, 239:1534-1536, 1988.
Ward et al. "Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*", Nature, vol. 341, pp. 544-546, 1989.
Waterhouse et al. "Combinatorial Infection and in Vivo Recombination: a Strategy for Making Large Phage Antibody Repertoires", Nuc. Acids. Res., 21:2265-2266, 1993.
Zhaolie, C. "Substitution of Asparagine for Glutamine for the Cultivation of Recombinant CHO Cell Line Producing Prourokinase"; Biotechnology Information, No. 2, pp. 25-27, published Feb. 28, 2001 (English translation).
Official Action received Apr. 30, 2014 in corresponding EP Application No. 10745064.5.
www.products.invitrogen.com/ivgn/product/Al049001; 2012.
International Search Report and Written Opinion mailed on Oct. 12, 2010, for PCT Application No. PCT/US2010/044795, filed on Aug. 8, 2010, 9 pages.

* cited by examiner

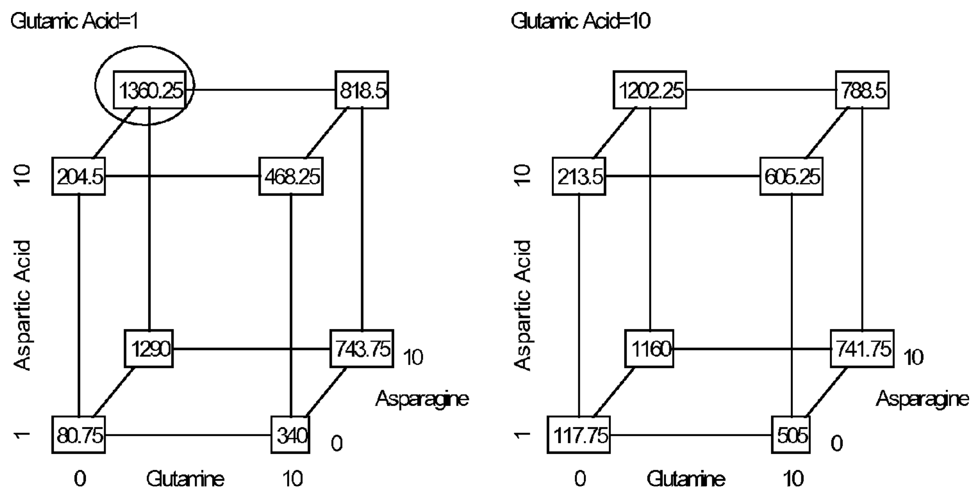
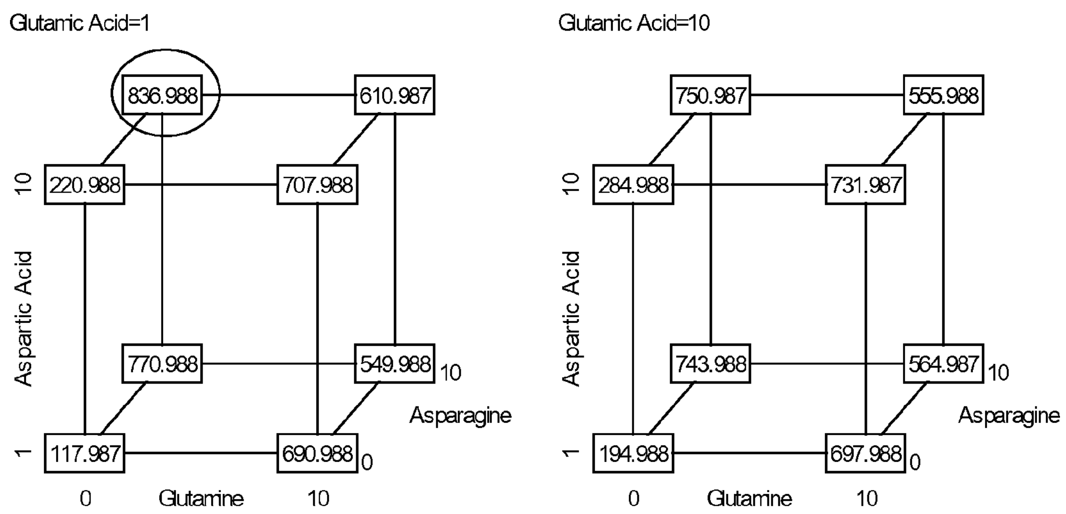


Figure 1.



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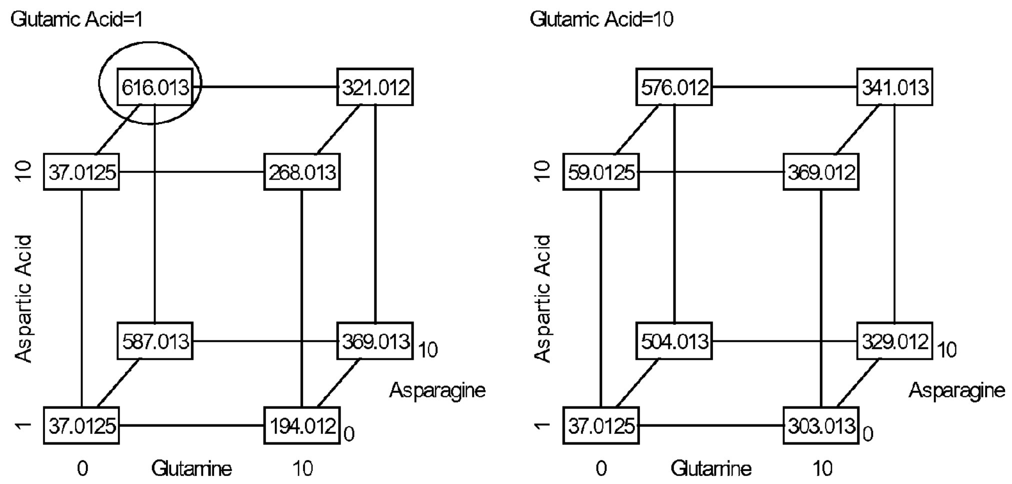

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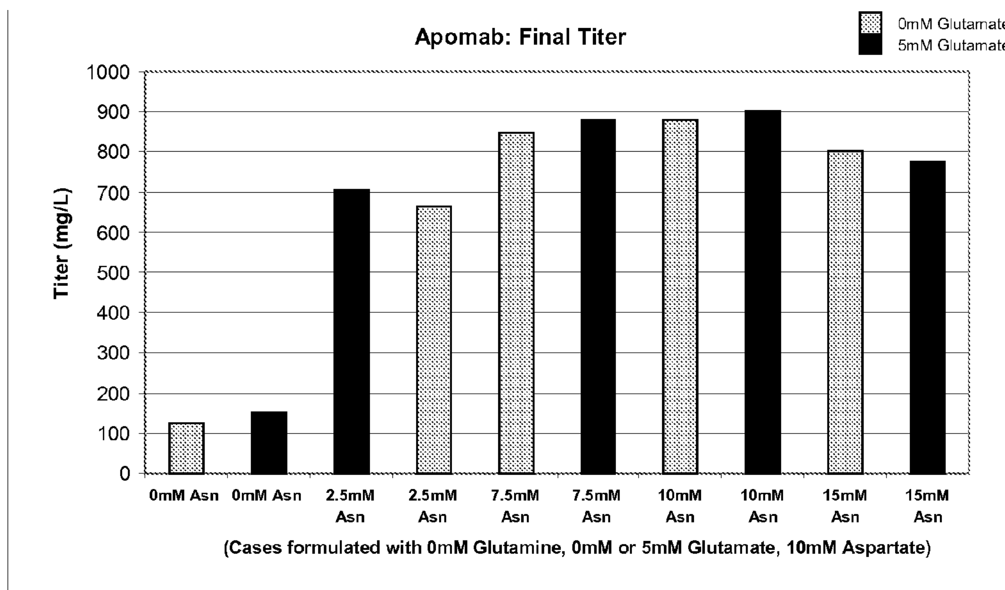
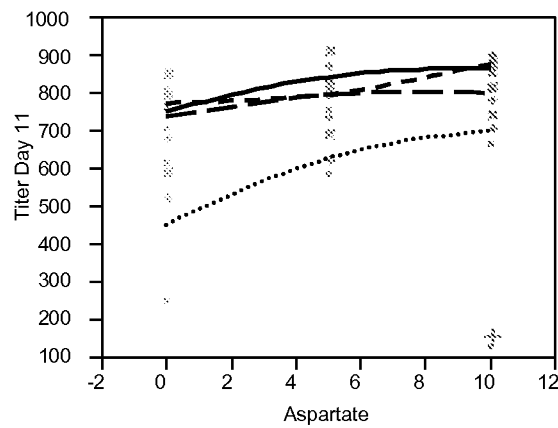


Figure 4.

Bivariate Fit of Titer Day 11 By Aspartate Glutamine=0



Smoothing Spline Fit, lambda=1 Asparagine==2.5
Smoothing Spline Fit, lambda=1 Asparagine==7.5
Smoothing Spline Fit, lambda=1 Asparagine==10
Smoothing Spline Fit, lambda=1 Asparagine==15

Smoothing Spline Fit, lambda=1 Asparagine==2.5

R-Square	0.573894
Sum of Squares Error	73461.63

Smoothing Spline Fit, lambda=1 Asparagine==7.5

R-Square	0.65596
Sum of Squares Error	11556.31

Smoothing Spline Fit, lambda=1 Asparagine==10

R-Square	0.408718
Sum of Squares Error	25684.65

Smoothing Spline Fit, lambda=1 Asparagine==15

R-Square	0.220438
Sum of Squares Error	26400.64

Figure 5.

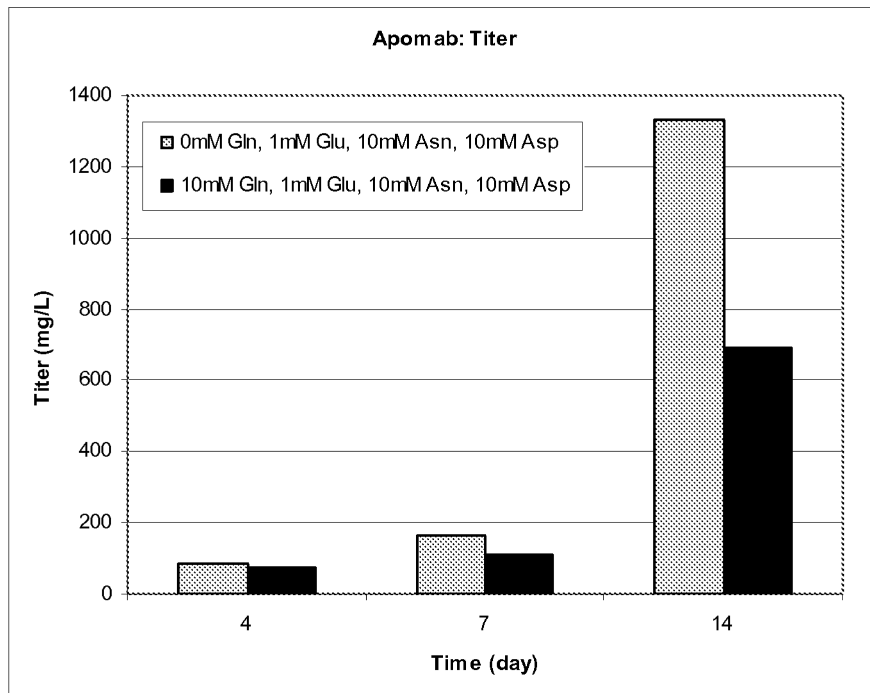


Figure 6A

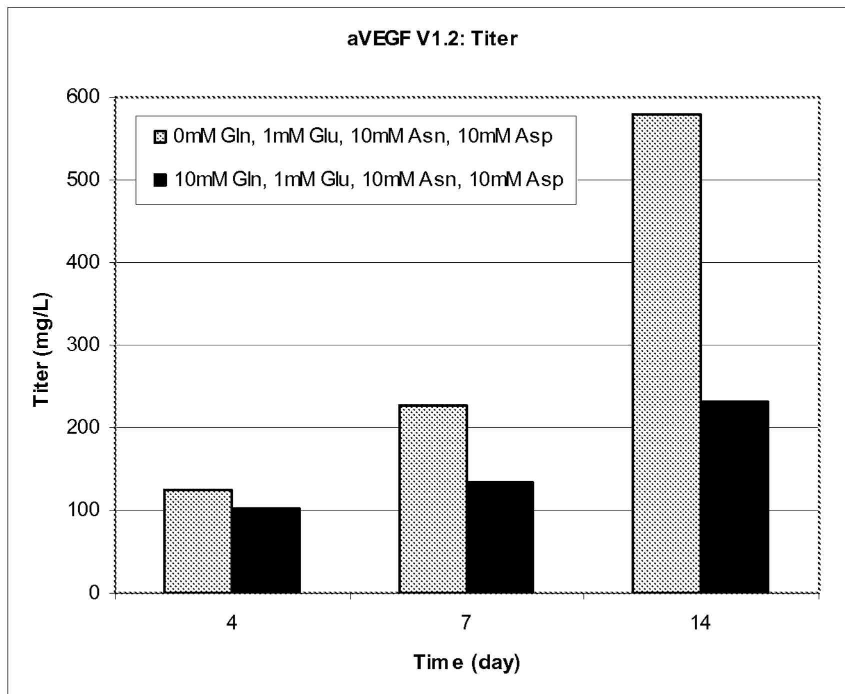


Figure 6B

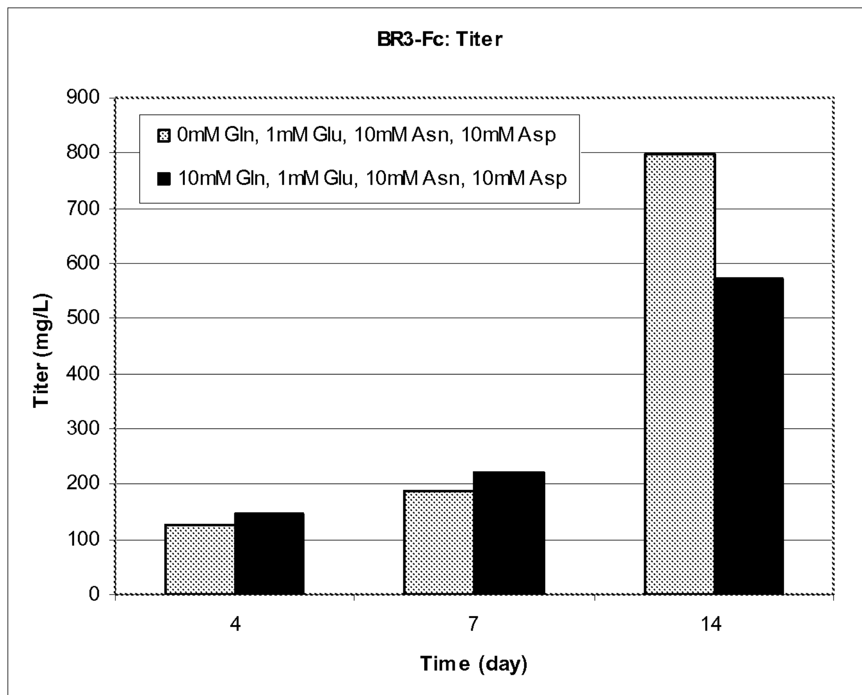


Figure 6C

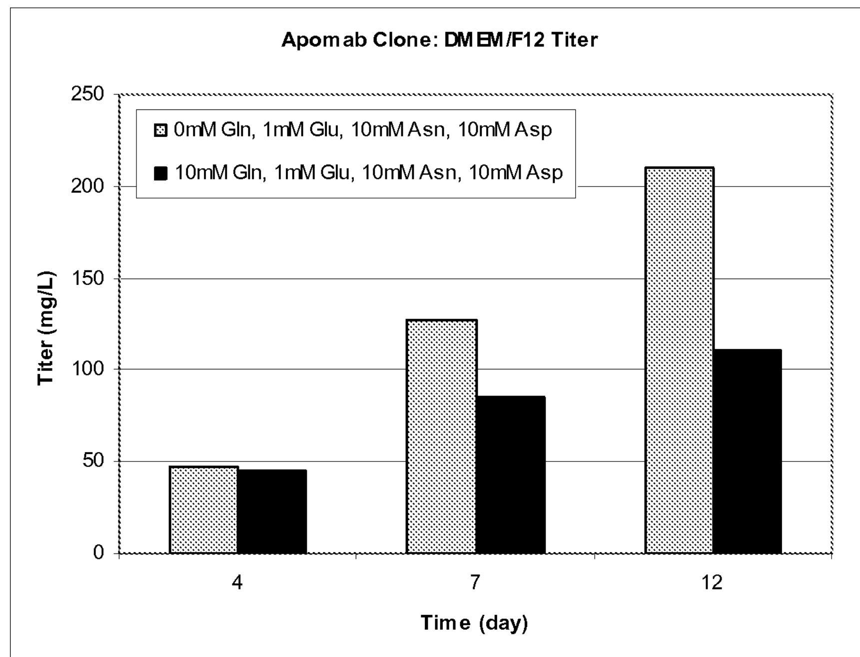


Figure 7A

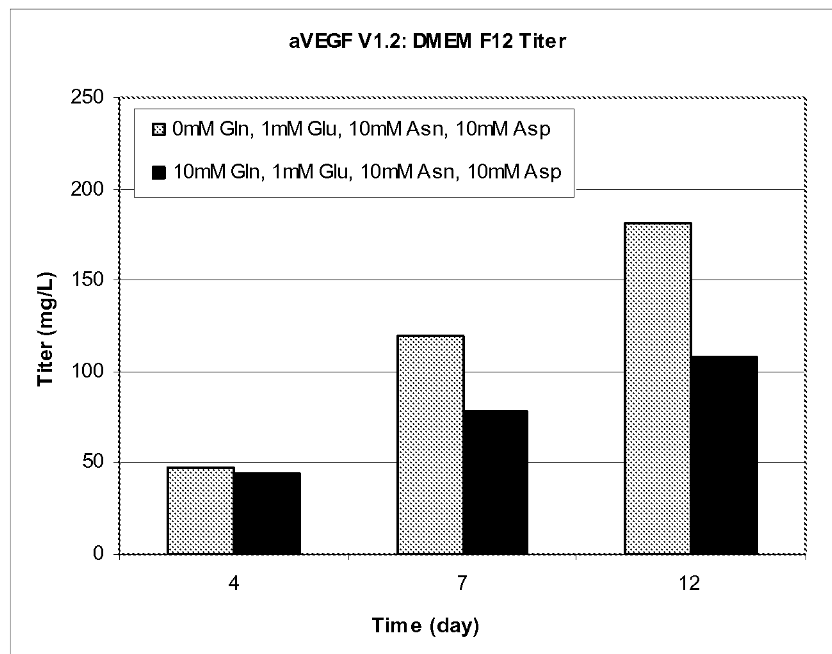


Figure 7B

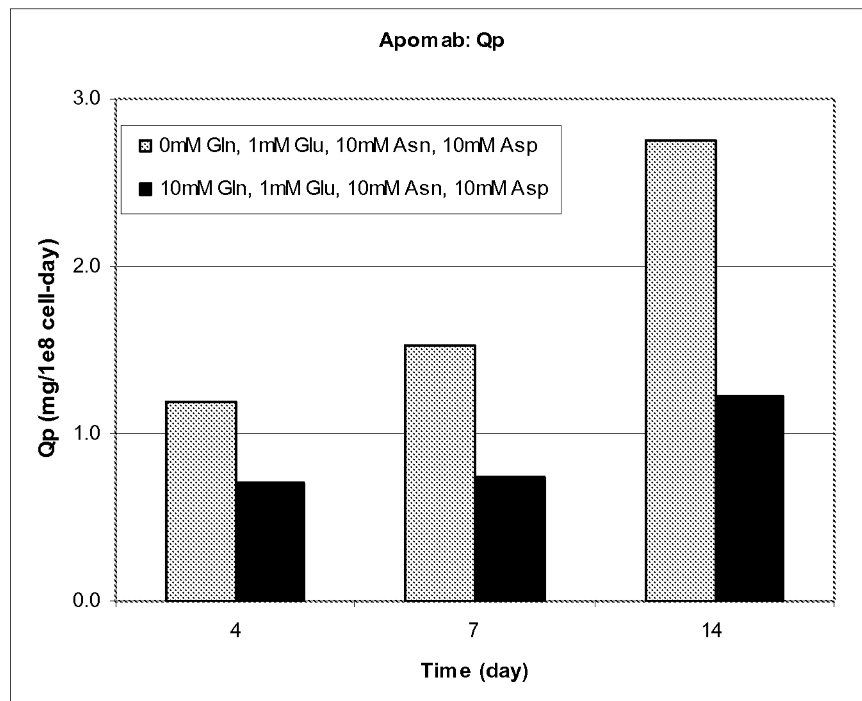
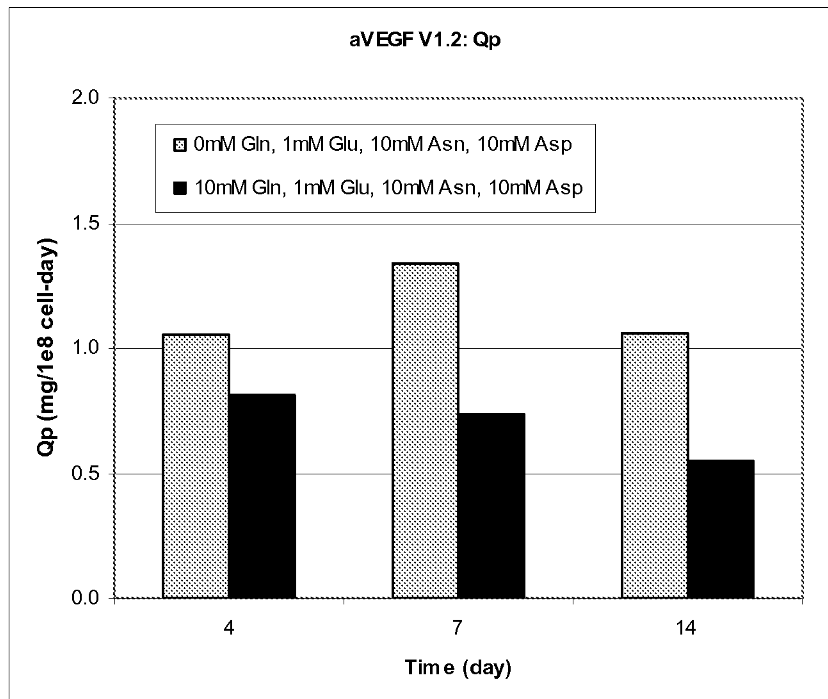


Figure 8A



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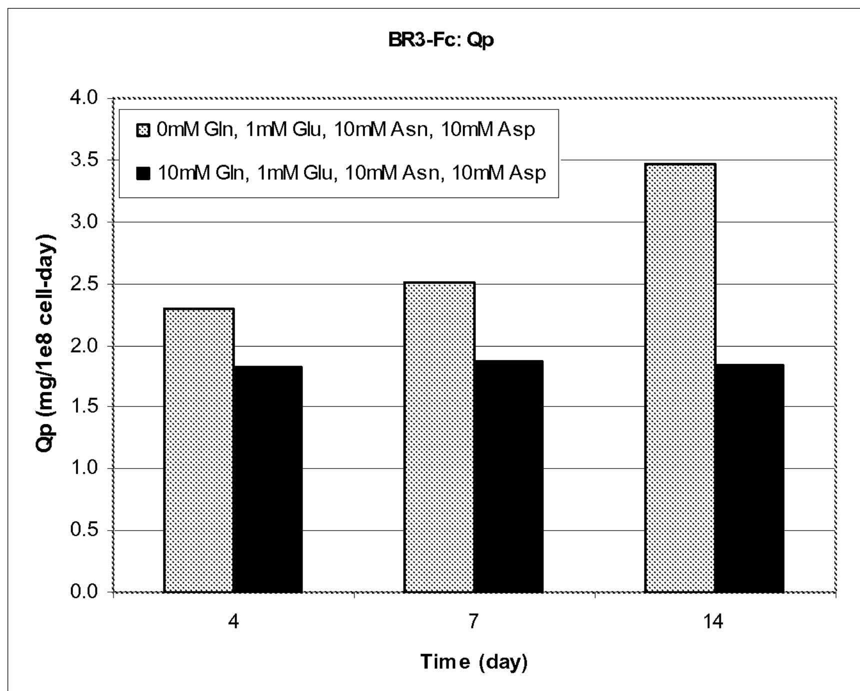


Figure 8C

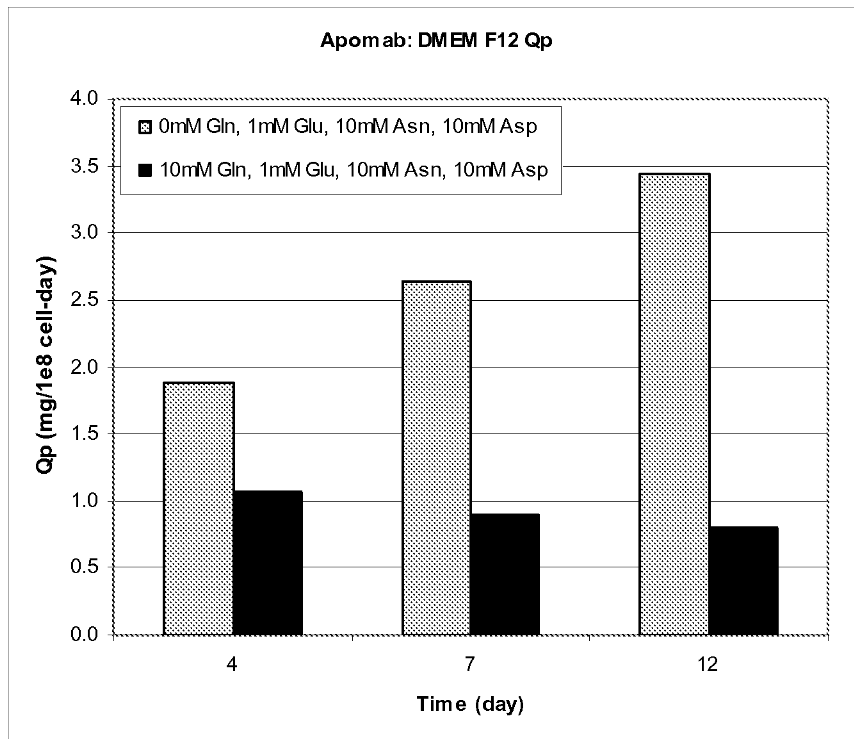
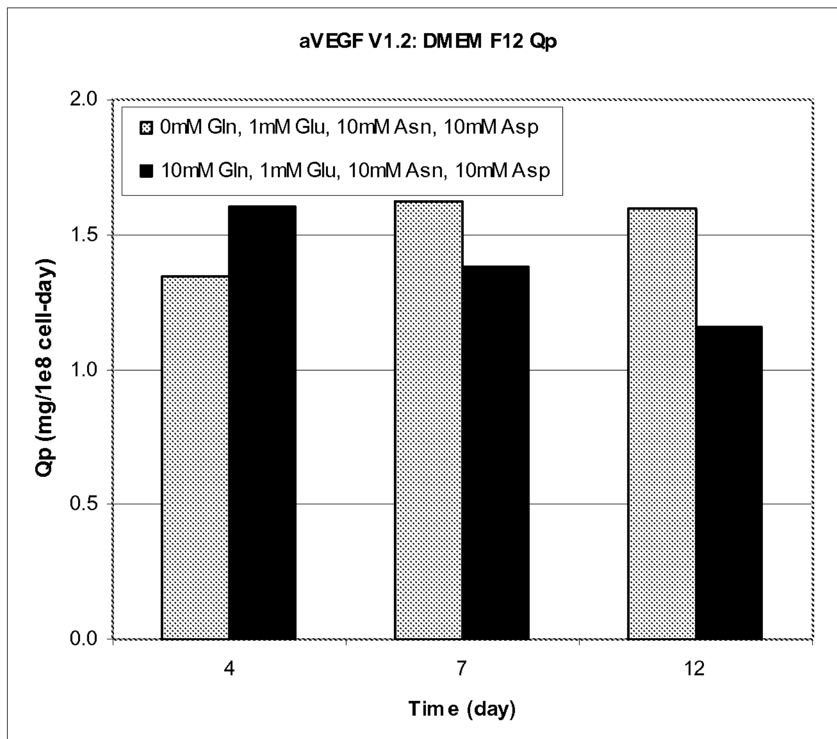


Figure 9A



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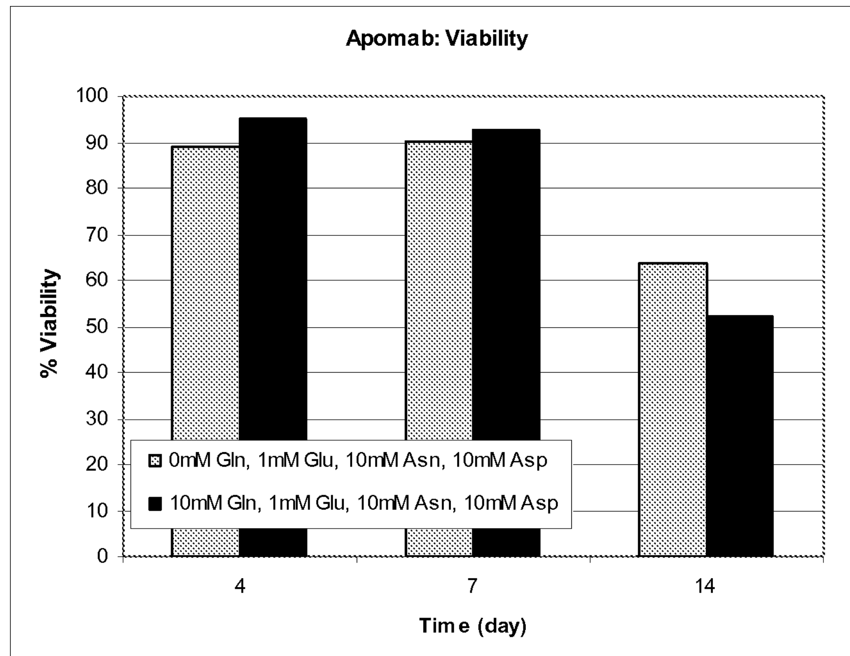
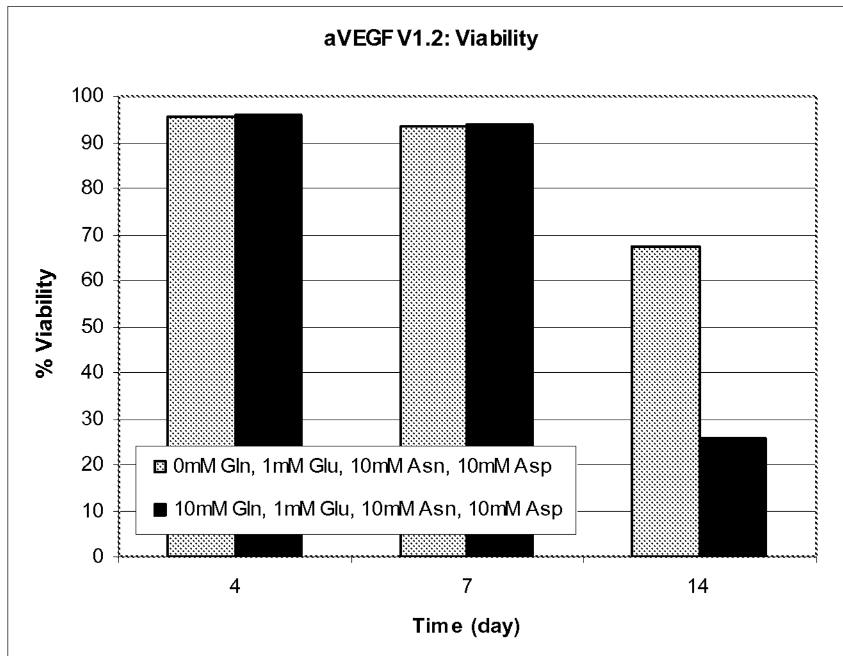


Figure 10A



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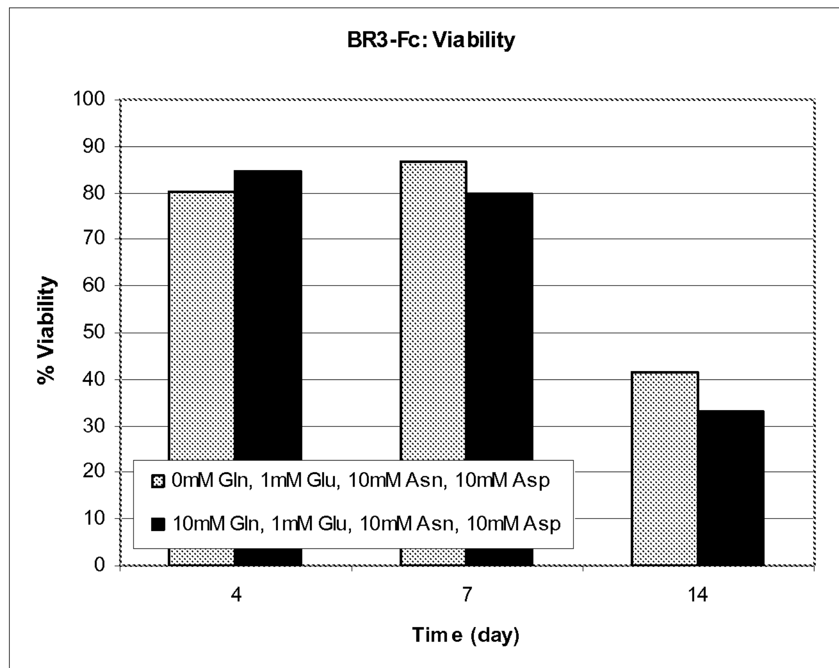

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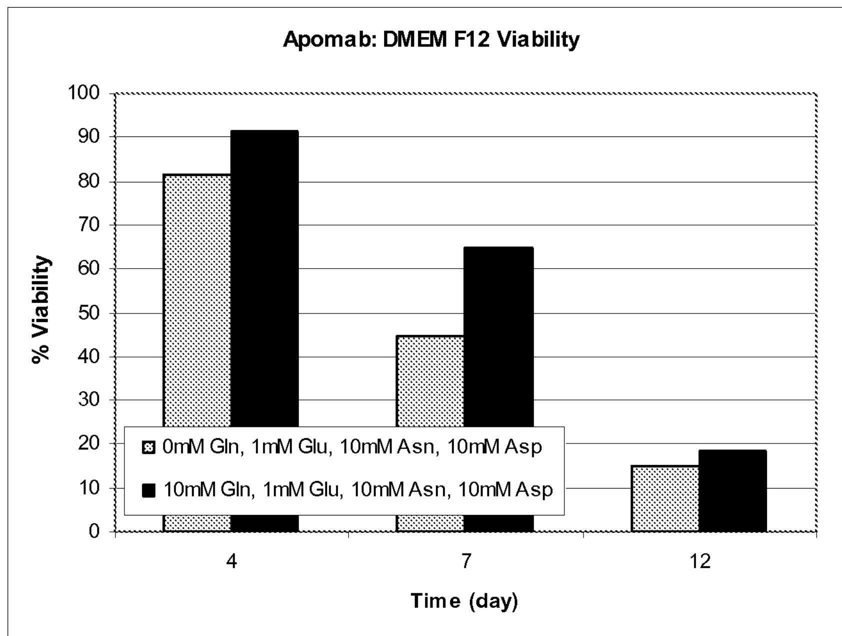
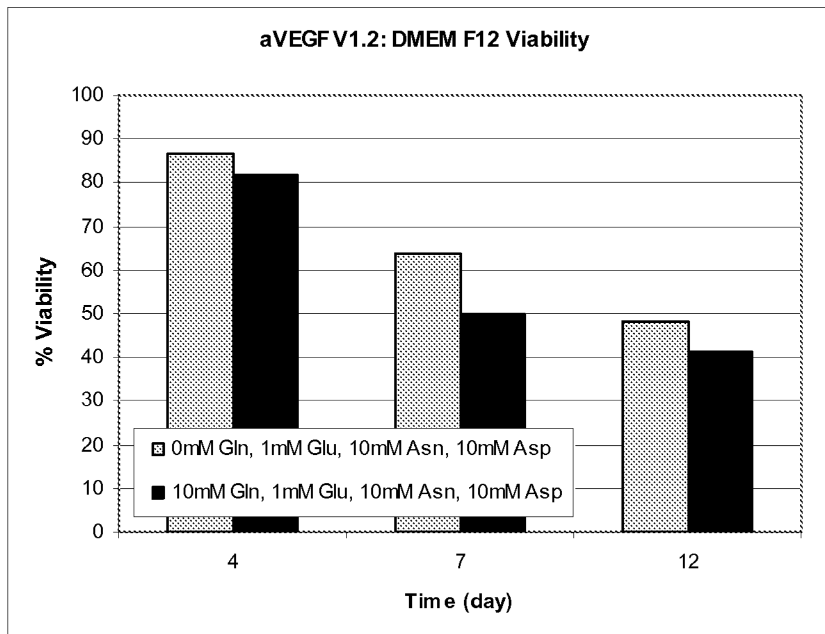


Figure 11A



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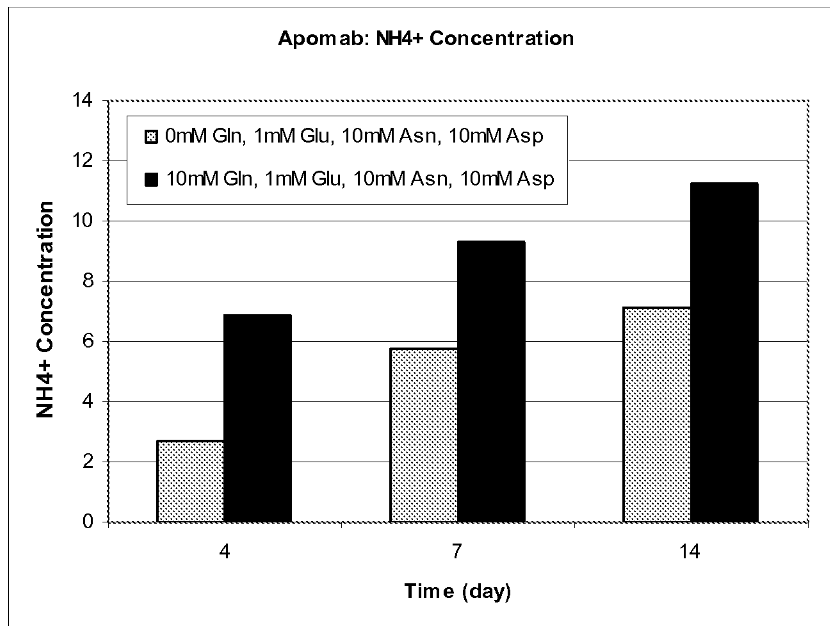

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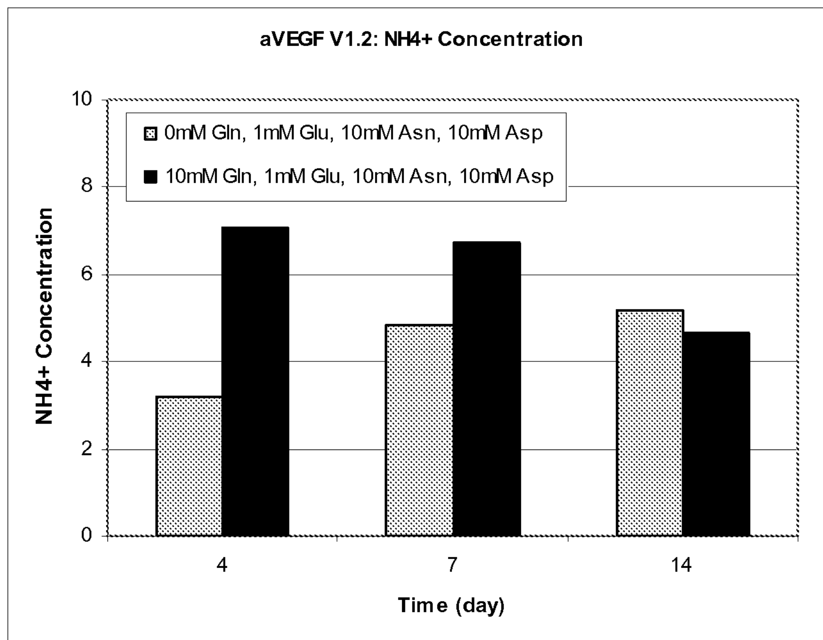

Figure 12B

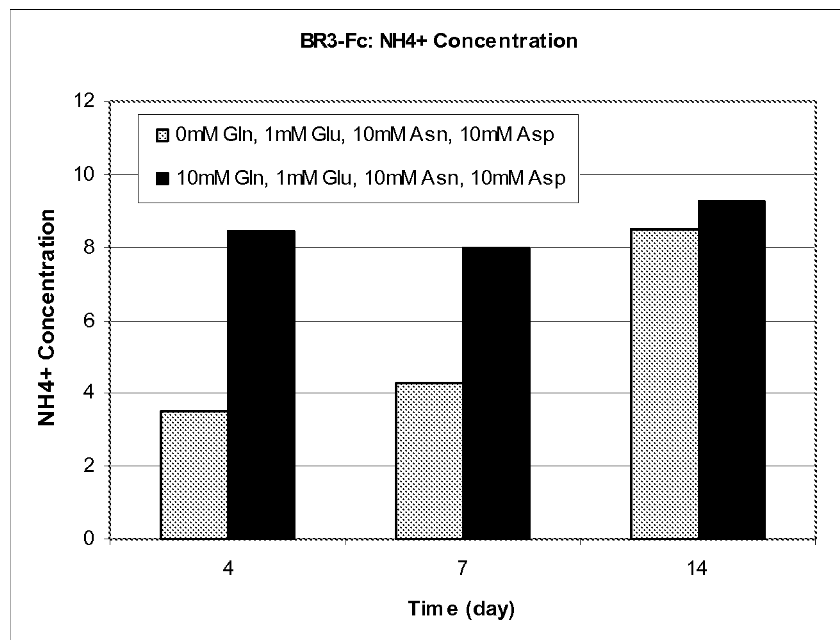

Figure 12C

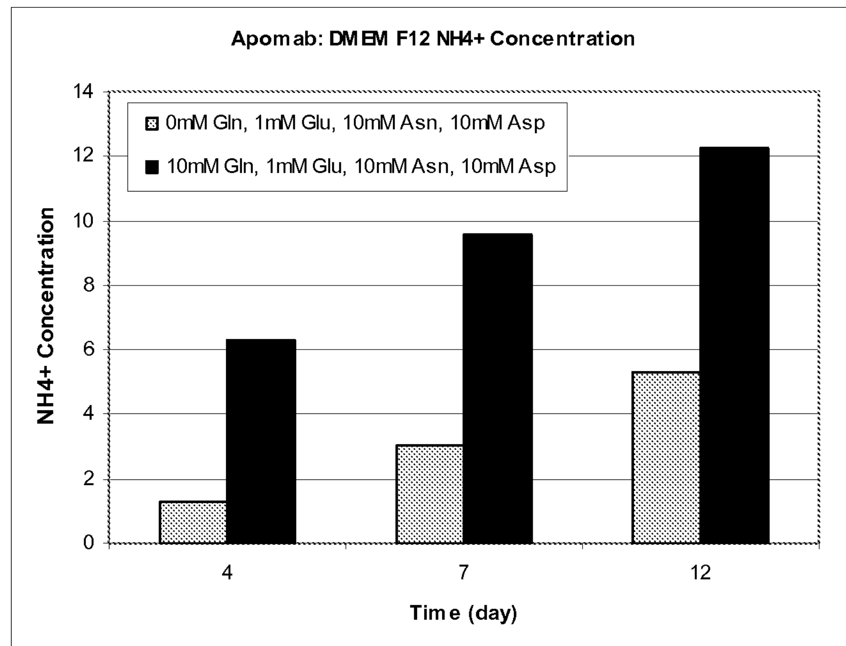

Figure 13A

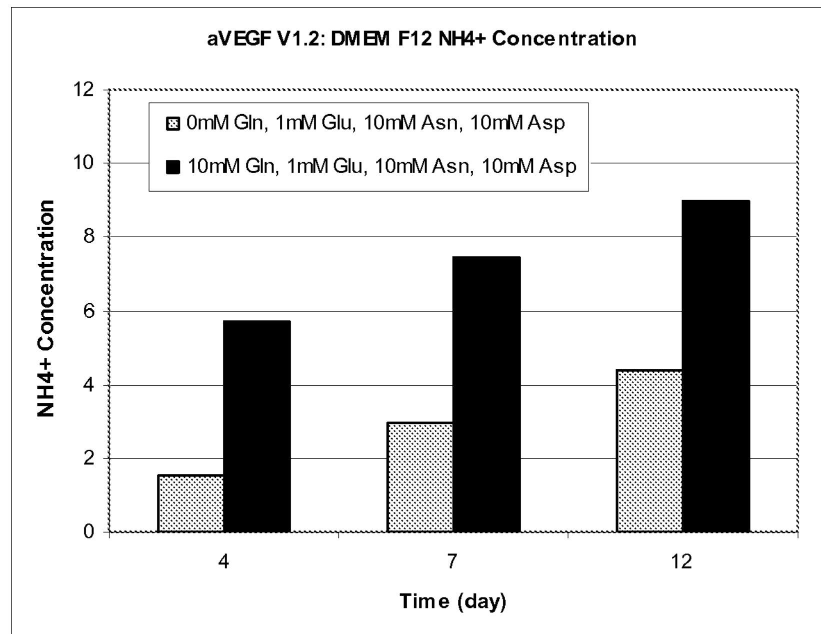

Figure 13B

PRODUCTION OF PROTEINS IN GLUTAMINE-FREE CELL CULTURE MEDIA

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 13/945,531, filed Jul. 18, 2013, now abandoned, which is a continuation of U.S. application Ser. No. 12/852,377, filed Aug. 6, 2010, now U.S. Pat. No. 8,512,983, issued Aug. 20, 2013, which claims priority under 35 USC §119(e) and the benefit of U.S. Provisional application No. 61/232,889, filed Aug. 11, 2009, the contents of which are incorporated herein by reference in their entireties.

BACKGROUND OF THE INVENTION

Mammalian cells have become the dominant system for the production of mammalian proteins for clinical applications, primarily due to their ability to produce properly folded and assembled heterologous proteins, and their capacity for post-translational modifications. It is conventional to have glutamine in cell culture media during recombinant production of heterologous proteins, including antibodies. L-glutamine is an essential amino acid, which is considered the primary energy and nitrogen sources for cells in culture. Most commercially available media are formulated with free L-glutamine which is either included in the basal formula or added to liquid media formulations at the time of use. Thus, all mammalian cell culture media contain glutamine except those for glutamine synthetase transfected cell lines, such as GS NS0 and GS CHO cell lines, where the cells themselves produce the glutamine needed for growth. Glutamine is widely used at various concentrations typically from 1 to 20 mM in base media and much higher concentration in feeds for fed-batch process. For example, the concentration of L-glutamine is 0.5 mM in Ames' Medium and 10 mM in MCDP Media 131. DMEM/Ham's Nutrient Mixture F-12 (50:50) is often used as a starting formulation for proprietary media used with Chinese Hamster Ovary (CHO) cells. L-glutamine in DMEM/Ham's Nutrient Mixture F-12 is 2.5 mM. L-glutamine concentration in Serum-Free/Protein Free Hybridoma Medium is 2.7 mM. L-glutamine in DMEM, GMEM, IMDM and H-Y medium is 4 mM, of which IMDM is often used as a starting formulation for proprietary hybridoma cell culture media. It is generally held that hybridoma cells grow better in concentrations of L-glutamine that are above the average levels found in media. (Dennis R. Conrad, Glutamine in Cell Culture, Sigma-Aldrich Media Expert)

It was shown that glutamine is the main source of ammonia accumulated in cell culture (see review by Markus Schneider, et. al. 1996, *Journal of Biotechnology* 46:161-185). Thus, lowering glutamine in cell culture media significantly reduced the accumulation of NH_4^+ level, resulting in lower cytotoxicity (see Markus Schneider, et. al. 1996, supra). Reduced NH_4^+ cytotoxicity resulted in higher cell viability, thus extended culture longevity. Based on an estimated glutamine consumption study using CHO cells, it was suggested that cells may consume glutamine at a rate of 0.3-0.4 mM per day (Miller, et. al. 1988, Biotechnol. Bioeng. 32: 947-965). Altamirano et al. (2001, *J. Biotechnol.* 110:171-9) studied the effect of glutamine replacement by glutamate and the balance between glutamate and glucose metabolism on the redistribution of CHO cells producing recombinant human tissue plasminogen activator (rhut-PA). When glutamine was replaced with glutamate and balanced

with glucose catabolism (carbon and nitrogen ratio, C/N ratio), cell metabolism was found redistributed and forced to utilize carbon and energy source more favorably to production of rhut-PA. It was also reported that CHO cells in adherent cultures can grow in the absence of added glutamine due to endogenous glutamine synthetase activity that allowed cells to synthesize glutamine from glutamic acid in the medium (Sanfeliu and Stephanopoulos, 1999, *Biotechnol. Bioeng.* 64:46-53). However, compared to control cultures in glutamine-containing media, the cell growth rate in glutamine-free media was slower with an increased fraction of cells distributed in the G0/G1 phase. The depletion of both glutamine and glutamic acid did cause cell death.

SUMMARY OF THE INVENTION

The present invention is based, at least in part, on the unexpected finding that not only can recombinant proteins be produced in a mammalian host cell using a glutamine-free production medium without any significant adverse effect, in fact the use of a glutamine-free medium in the production phase significantly increases cell viability, culture longevity, specific productivity and/or the final recombinant protein titer.

The present invention is also based on the unexpected finding that the addition of asparagine to a glutamine-free production medium can further enhance the cell viability, culture longevity, specific productivity and/or the final recombinant protein titer in a mammalian host cell using a glutamine-free production medium without any significant adverse effect.

In one aspect, the invention concerns a process for producing a polypeptide in a mammalian host cell expressing said polypeptide, comprising culturing the mammalian host cell in a production phase of the culture in a glutamine-free production culture medium supplemented with asparagine.

In one embodiment, the mammalian host cell is a Chinese Hamster Ovary (CHO) cell.

In another embodiment, the mammalian host cell is a $dhfr^-$ CHO cell.

In yet another embodiment, the production medium is serum-free.

In a further embodiment, the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

In a still further embodiment, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

In all embodiments, the production phase may, for example, be a batch or fed batch culture phase.

In all embodiments, the process may further comprise the step of isolating said polypeptide.

In a further embodiment, isolation may be followed by determining one or more of cell viability, culture longevity, specific productivity and final recombinant protein titer following isolation.

In a still further embodiment, at least one of the cell viability, culture longevity, specific productivity and final recombinant protein titer is increased relative to the same

polypeptide produced in a glutamine-containing production medium of the same composition.

In a further aspect, the invention concerns a ready-to-use glutamine-free cell culture medium for the production of a polypeptide in a production phase.

In yet another embodiment, the polypeptide is a mammalian glycoprotein.

In other embodiments, the polypeptide is selected from the group consisting of antibodies, antibody fragments, and immunoadhesins.

In all embodiments, the polypeptide may, for example, be an antibody, or a biologically functional fragment of an antibody. Representative antibody fragments include Fab, Fab', $F(ab)_2$, scFv, $(scFv)_2$, dAb, complementarity determining region (CDR) fragments, linear antibodies, single-chain antibody molecules, minibodies, diabodies, and multispecific antibodies formed from antibody fragments.

In a still further embodiment, the antibody or antibody fragment is chimeric, humanized or human.

Therapeutic antibodies include, without limitation, anti-HER2 antibodies anti-CD20 antibodies; anti-IL-8 antibodies; anti-VEGF antibodies; anti-CD40 antibodies, anti-CD11a antibodies; anti-CD18 antibodies; anti-IgE antibodies; anti-Apo-2 receptor antibodies; anti-Tissue Factor (TF) antibodies; anti-human $\alpha_4\beta_7$ integrin antibodies; anti-EGFR antibodies; anti-CD3 antibodies; anti-CD25 antibodies; anti-CD4 antibodies; anti-CD52 antibodies; anti-Fc receptor antibodies; anti-carcinoembryonic antigen (CEA) antibodies; antibodies directed against breast epithelial cells; antibodies that bind to colon carcinoma cells; anti-CD38 antibodies; anti-CD33 antibodies; anti-CD22 antibodies; anti-EpCAM antibodies; anti-GpIIb/IIIa antibodies; anti-RSV antibodies; anti-CMV antibodies; anti-HIV antibodies; anti-hepatitis antibodies; anti-CA 125 antibodies; anti-$\alpha v\beta 3$ antibodies; anti-human renal cell carcinoma antibodies; anti-human 17-1A antibodies; anti-human colorectal tumor antibodies; anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma; and anti-human leukocyte antigen (HLA) antibodies, and anti-HLA DR antibodies.

In other embodiments, the therapeutic antibody is an antibody binding to a HER receptor, VEGF, IgE, CD20, CD11a, CD40, or DR5.

In other embodiments, the therapeutic antibody is an anti-BR3 antibody or BR3-Fc immunoadhesin.

In other embodiments of the method of the present invention, the polypeptide expressed in the recombinant host cell is a therapeutic polypeptide. For example, the therapeutic polypeptide can be selected from the group consisting of a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of said polypeptides.

In all embodiments, the recombinant host cell can be an eukaryotic host cell. such as a mammalian host cell, including, for example, Chinese Hamster Ovary (CHO) cells.

These and other aspects will be apparent from the description below, including the Examples and the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Apomab antibody cube plot analysis of titer results from a Full Factorial Design of Experiment (DOE) evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate. The model predicts that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

FIG. **2**. BR3-Fc immunoadhesin cube plot analysis of titer results from a Full Factorial DOE evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate. The model predicts that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

FIG. **3**. anti-VEGF antibody cube plot analysis of titer results from a Full Factorial DOE evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate. The model predicts that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

FIG. **4**. Effect of Asparagine under Glutamine-free, low Glutamate and high Aspartate conditions on Apomab antibody titer. In Glutamine-free medium, Apomab antibody titer was significantly increased in the presence of 2.5-15 mM Asparagine compared to Glutamine-free cultures without Asparagine. Under these conditions, the presence or absence of Glutamate had no effect on titer.

FIG. **5**. Apomab antibody titer production across various Asparagine and Aspartate concentrations in Glutamine-free

and low Glutamate conditions. A positive titration effect was observed when increasing Aspartate from 0 to 10 mM under these conditions.

FIGS. **6**. A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on titer. The final titer for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin was significantly higher in Glutamine-free medium compared to Glutamine-containing medium.

FIGS. **7**-A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on titer. The final titer for Apomab antibody and anti-VEGF antibody was significantly higher in Glutamine-free DMEM/F12 medium compared to Glutamine-containing DMEM F12 medium.

FIGS. **8** A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on cell specific productivity (Qp). Cell specific productivity for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin was significantly higher in Glutamine-free medium compared to Glutamine-containing medium.

FIGS. **9** A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on cell specific productivity (Qp). Cell specific productivity for Apomab antibody and anti-VEGF antibody was significantly higher in Glutamine-free DMEM/F12 medium compared to Glutamine-containing DMEM/F12 medium.

FIGS. **10** A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on Cell Viability. Cell viability for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin was higher in Glutamine-free medium compared to Glutamine-containing medium.

FIGS. **11** A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on Cell Viability. In DMEM/F12 medium, cell viability was not consistently improved in Glutamine-free medium. Viability was higher for Apomab antibody, but lower for anti-VEGF antibody compared to Glutamine containing medium.

FIGS. **12** A-C. Effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on ammonia formation Ammonia was usually lower in Glutamine-free cultures compared to Glutamine-containing cultures.

FIGS. **13** A and B. Effect of DMEM/F12 glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on ammonia formation. Ammonia was significantly reduces in Glutamine-free DMEM/F12 medium compared to Glutamine-containing DMEM/F12 medium.

DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

The terms “cell culture medium”, “culture medium”, and “nutrient mixture” refer to a nutrient solution used for growing mammalian cells that typically provides at least one component from one or more of the following categories:

1) an energy source, usually in the form of a carbohydrate such as glucose;

2) some or all of the essential amino acids, and usually the basic set of twenty amino acids plus cystine;

3) vitamins and/or other organic compounds typically required at low concentrations;

4) free fatty acids; and

5) trace elements, where trace elements are defined as inorganic compounds or naturally occurring elements that are typically required at very low concentrations, usually in the micromolar range.

The nutrient mixture may optionally be supplemented with one or more component from any of the following categories:

1) hormones and other growth factors as, for example, insulin, transferrin, and epidermal growth factor;

2) salts and buffers as, for example, calcium, magnesium, and phosphate; and

3) nucleosides such as, for example, adenosine and thymidine.

The cell culture medium is generally “serum free” when the medium is essentially free of serum from any mammalian source (e.g. fetal bovine serum (FBS)). By “essentially free” is meant that the cell culture medium comprises between about 0-5% serum, preferably between about 0-1% serum, and most preferably between about 0-0.1% serum. Advantageously, serum-free “defined” medium can be used, wherein the identity and concentration of each of the components in the medium is known (i.e., an undefined component such as bovine pituitary extract (BPE) is not present in the culture medium).

In the context of the present invention the expressions “cell”, “cell line”, and “cell culture” are used interchangeably, and all such designations include progeny. Thus, the words “transformants” and “transformed (host) cells” include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

The term “animal host cell,” “animal cell,” “animal recombinant host cell,” and the like, encompasses invertebrate, non-mammalian vertebrate (e.g., avian, reptile and amphibian) and mammalian cells. Examples of invertebrate cells include the following insect cells: *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori*. See, e.g., Luckow et al., Bio/Technology, 6:47-55 (1988); Miller et al., in *Genetic Engineering*, Setlow, J. K. et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., *Nature,* 315:592-594 (1985).

The term “mammalian host cell,” “mammalian cell,” “mammalian recombinant host cell,” and the like, refer to cell lines derived from mammals that are capable of growth and survival when placed in either monolayer culture or in suspension culture in a medium containing the appropriate nutrients and growth factors. The necessary nutrients and growth factors for a particular cell line are readily determined empirically without undue experimentation, as described for example in *Mammalian Cell Culture* (Mather, J. P. ed., Plenum Press, N.Y. (1984)), and by Barnes and Sato (*Cell,* 22:649 (1980)). Typically, the cells are capable of expressing and secreting large quantities of a particular protein of interest (typically a recombinant protein) into the culture medium, and are cultured for this purpose. However, the cells may be cultured for a variety of other purposes as well, and the scope of this invention is not limited to culturing the cells only for production of recombinant pro-

teins. Examples of suitable mammalian cell lines, capable of growth in the media of this invention, include monkey kidney CVI line transformed by SV40 (COS-7, ATCC® CRL 1651); human embryonic kidney line 293S (Graham et al., *J. Gen. Virolo.,* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC® CCL 10); mouse sertoli cells (TM4, Mather, *Biol. Reprod.,* 23:243 (1980)); monkey kidney cells (CVI-76, ATCC® CCL 70); African green monkey kidney cells (VERO-76, ATCC® CRL-1587); human cervical carcinoma cells (HELA, ATCC® CCL 2); canine kidney cells (MDCK, ATCC® CCL 34); buffalo rat liver cells (BRL 3A, ATCC® CRL 1442); human lung cells (W138, ATCC® CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor cells (MMT 060562, ATCC® CCL 5I); rat hepatoma cells (HTC, MI.54, Baumann et al., J. Cell Biol., 85:1 (1980)); and TR-1 cells (Mather et al., *Annals N.Y. Acad. Sci.,* 383:44 (1982)) and hybridoma cell lines. Chinese hamster ovary cells (Urlab and Chasin, *Proc. Natl. Acad. Sci. USA,* 77:4216 (1980)) are a preferred cell line for practicing this invention. CHO cells suitable for use in the methods of the present invention have also been described in the following documents: EP 117,159, published Aug. 29, 1989; U.S. Pat. Nos. 4,766,075; 4,853,330; 5,185,259; Lubiniecki et al., in *Advances in Animal Cell Biology and Technology for Bioprocesses*, Spier et al., eds. (1989), pp. 442-451. Known CHO derivatives suitable for use herein include, for example, CHO/−DHFR (Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA,* 77: 4216 (1980)), CHO-K1 DUX B11 (Simonsen and Levinson, *Proc. Natl. Acad. Sci. USA* 80: 2495-2499 (1983); Urlaub and Chasin, supra), and dp 12.CHO cells (EP 307,247 published Mar. 15, 1989). Preferred host cells include CHO-K1 DUX B11 and dp 12.CHO cells.

"dhfr$^-$ CHO cell" refers to a dihydrofolate reductase (DHFR) deficient CHO cell. Production of recombinant proteins in mammalian cells has allowed the manufacture of a number of large, complex glycosylated polypeptides for clinical applications. Chinese hamster ovary (CHO) DHFR− cells and the amplifiable selectable marker DHFR are routinely used to establish cell lines that produce clinically useful amounts of product. (Urlab, G. and Chasin, L. A. (1980) Proc. Natl Acad. Sci. USA, 77, 4216-4220; Kaufman, R. J. and Sharp, P. (1982) J. Mol. Biol., 159, 601-621; Gasser, C. S., Simonsen, C. S., Schilling, J. W. and Schmike, R. T. (1982) Proc. Natl Sci. USA, 79, 6522-6526)

By "phase" is meant a certain phase of culturing of the cells as is well recognized by the practitioner.

"Growth phase" of the cell culture refers to the period of exponential cell growth (the log phase) where cells are generally rapidly dividing. During this phase, cells are cultured for a period of time, usually between 1-4 days, and under such conditions that cell growth is maximized. The growth cycle for the host cell can be determined for the particular host cell envisioned without undue experimentation. During the growth phase, cells are cultured in nutrient medium containing the necessary additives generally at about 30-40° C., preferably about 37° C., in a humidified, controlled atmosphere, such that optimal growth is achieved for the particular cell line. Cells are maintained in the growth phase for a period of between about one and four days, usually between about two and three days.

"Transition phase" of the cell culture refers to the period of time during which culture conditions for the production phase are engaged. During the transition phase environmental factors such as temperature are shifted from growth conditions to production conditions.

"Production phase" of the cell culture refers to the period of time during which cell growth has plateaued. During the production phase, logarithmic cell growth has ended and protein production is primary. During this period of time the medium is generally supplemented to support continued protein production and to achieve the desired protein product.

The phrase "fed batch cell culture" when used herein refers to a batch culture wherein the animal (e.g. mammalian) cells and culture medium are supplied to the culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. Fed batch culture includes "semi-continuous fed batch culture" wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium. Fed batch culture is distinguished from simple "batch culture" in which all components for cell culturing (including the animal cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed batch culture can be further distinguished from perfusion culturing insofar as the supernatant is not removed from the culturing vessel during the process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers etc and the culture medium is continuously or intermittently introduced and removed from the culturing vessel). However, removal of samples for testing purposes during fed batch cell culture is contemplated.

When used herein, the term "glutamine" refers to the amino acid L-glutamine (also known as "Gln" and "Q" by three-letter and single-letter designation, respectively) which is recognized as both an amino acid building block for protein synthesis and as an energy source in cell culture. Thus, the terms "glutamine" and "L-glutamine" are used interchangeably herein.

The word "glucose" refers to either of α-D-glucose or β-D-glucose, separately or in combination. It is noted that α and β glucose forms are interconvertible in solution.

The expression "osmolality" is a measure of the osmotic pressure of dissolved solute particles in an aqueous solution. The solute particles include both ions and non-ionized molecules. Osmolality is expressed as the concentration of osmotically active particles (i.e., osmoles) dissolved in 1 kg of water (1 mOsm/kg H_2O at 38° C. is equivalent to an osmotic pressure of 19 mm Hg). "Osmolarity" refers to the number of solute particles dissolved in 1 liter of solution. Solutes which can be added to the culture medium so as to increase the osmolality thereof include proteins, peptides, amino acids, non-metabolized polymers, vitamins, ions, salts, sugars, metabolites, organic acids, lipids, etc. In the preferred embodiment, the concentration of amino acids and NaCl in the culture medium is increased in order to achieve the desired osmolality ranges set forth herein. When used herein, the abbreviation "mOsm" means "milliosmoles/kg H_2O".

The term "cell density" as used herein refers to that number of cells present in a given volume of medium.

The term "cell viability" as used herein refers to the ability of cells in culture to survive under a given set of culture conditions or experimental variations. The term as used herein also refers to that portion of cells which are alive at a particular time in relation to the total number of cells, living and dead, in the culture at that time.

The terms "amino acids" and "amino acid" refer to all naturally occurring alpha amino acids in both their D and L stereoisomeric forms, and their analogs and derivatives. An

analog is defined as a substitution of an atom in the amino acid with a different atom that usually has similar properties. A derivative is defined as an amino acid that has another molecule or atom attached to it. Derivatives would include, for example, acetylation of an amino group, amination of a carboxyl group, or oxidation of the sulfur residues of two cysteine molecules to form cystine.

The term "protein" is meant to refer to a sequence of amino acids for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. This is to distinguish from "peptides" or other small molecular weight drugs that do not have such structure. Typically, the protein herein will have a molecular weight of at least about 15-20 kD, preferably at least about 20 kD. Examples of proteins encompassed within the definition herein include all mammalian proteins, in particular, therapeutic and diagnostic proteins, such as therapeutic and diagnostic antibodies, and, in general proteins that contain one or more disulfide bonds, including multi-chain polypeptides comprising one or more inter- and/or intrachain disulfide bonds.

The term "therapeutic protein" or "therapeutic polypeptide" refers to a protein that is used in the treatment of disease, regardless of its indication or mechanism of action. In order for therapeutic proteins to be useful in the clinic it must be manufactured in large quantities. "Manufacturing scale" production of therapeutic proteins, or other proteins, utilize cell cultures ranging from about 400 L to about 80,000 L, depending on the protein being produced and the need. Typically such manufacturing scale production utilizes cell culture sizes from about 400 L to about 25,000 L. Within this range, specific cell culture sizes such as 4,000 L, about 6,000 L, about 8,000, about 10,000, about 12,000 L, about 14,000 L, or about 16,000 L are utilized.

As used herein, "polypeptide of interest" refers generally to peptides and proteins having more than about ten amino acids. The polypeptides may be homologous to the host cell, or preferably, may be exogenous, meaning that they are heterologous, i.e., foreign, to the host cell being utilized, such as a human protein produced by a non-human mammalian, e.g., Chinese Hamster Ovary (CHO) cell. Preferably, mammalian polypeptides (polypeptides that were originally derived from a mammalian organism) are used, more preferably those which are directly secreted into the medium. The term "polypeptide" or "polypeptide of interest" specifically includes antibodies, in particular, antibodies binding to mammalian polypeptides, such as any of the mammalian polypeptides listed below or fragments thereof, as well as immunoadhesins (polypeptide-Ig fusion), such as those comprising any of the mammalian polypeptides listed below, or fragments thereof.

Examples of mammalian polypeptides include, without limitation, transmembrane molecules (e.g. receptors) and ligands such, as growth factors. Exemplary polypeptides include molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; interferon such as interferon-α, -β, and -γ; lipoproteins; α-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA), including t-PA variants; bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as β-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER1 (EGFR), HER2, HER3 or HER4 receptor; Apo2L/TRAIL, hedgehog, mitogen activated protein kinase (MAPK), and fragments of any of the above-listed polypeptides. Apo2L (TRAIL) and is variants are disclosed, for example, in U.S. Application Publication No. 20040186051. Anti-VEGF antibodies are disclosed, for example, in U.S. Pat. Nos. 8,994,879; 7,060,269; 7,169,901; and 7,297,334. Anti-CD20 antibodies are disclosed, for example, in U.S. Application Publication No. 20060246004. The BR3 polypeptide, anti-BR3 antibodies and BR3-Fc immunoadhesins are described, for example, in U.S. Application Publication No. 20050070689.

As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the binding specificity of a heterologous protein (an "adhesin") with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of an amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site of an antibody (i.e., is "heterologous"), and an immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding site of a receptor or a ligand. The immunoglobulin constant domain sequence in the immunoadhesin may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA (including IgA-1 and IgA-2), IgE, IgD or IgM.

As noted above, in certain embodiments, the protein is an antibody. "Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which generally lack antigen speci-

ficity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region or intact monoclonal antibodies), antibody compositions with polyepitopic specificity, polyclonal antibodies, multivalent antibodies, multispecific antibodies (e.g., bispecific antibodies) formed from at least two intact antibodies, diabodies, and single-chain molecules such as scFv molecules, as well as antibody fragments (e.g., Fab, F(ab')2, and Fv).

Unless indicated otherwise, the expression "multivalent antibody" is used throughout this specification to denote an antibody comprising three or more antigen binding sites. The multivalent antibody is typically engineered to have the three or more antigen binding sites and is generally not a native sequence IgM or IgA antibody.

The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain the Fc region.

"Antibody fragments" comprise only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such an antibody fragment may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')2 fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')2 antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known. Examples of antibody fragments encompassed by the present definition include: (i) the Fab fragment, having VL, CL, VH and CH1 domains; (ii) the Fab' fragment, which is a Fab fragment having one or more cysteine residues at the C-terminus of the CH1 domain; (iii) the Fd fragment having VH and CH1 domains; (iv) the Fd' fragment having VH and CH1 domains and one or more cysteine residues at the C-terminus of the CH1 domain; (v) the Fv fragment having the VL and VH domains of a single arm of an antibody; (vi) the dAb fragment (Ward et al., *Nature* 341, 544-546 (1989)) which consists of a VH domain; (vii) isolated CDR regions; (viii) F(ab')2 fragments, a bivalent fragment including two Fab' fragments linked by a disulphide bridge at the hinge region; (ix) single chain antibody molecules (e.g. single chain Fv; scFv) (Bird et al., *Science* 242:423-426 (1988); and Huston et al., *PNAS* (*USA*) 85:5879-5883 (1988)); (x) "diabodies" with two antigen binding sites, comprising a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (see, e.g., EP 404,097; WO 93/11161; and Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993)); (xi) "linear antibodies" comprising a pair of tandem Fd segments (VH-CH1-VH-CH1) which, together with complementary light chain polypeptides, form a pair of antigen binding regions (Zapata et al. *Protein Eng.* 8(10):1057 1062 (1995); and U.S. Pat. No. 5,641,870).

"Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

"Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404,097; WO93/1161; Hudson et al., (2003) Nat. Med. 9:129-134; and Hollinger et al., Proc. Natl. Acad. Sci. USA 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., (2003) Nat. Med. 9:129-134.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible mutations, e.g., naturally occurring mutations, that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. Monoclonal antibodies are highly specific, being directed against a single antigen. In certain

embodiments, a monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins.

The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler and Milstein, *Nature,* 256:495-97 (1975); Hongo et al., *Hybridoma,* 14 (3): 253-260 (1995), Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567), phage-display technologies (see, e.g., Clackson et al., *Nature,* 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1991); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101(34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284 (1-2): 119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes encoding human immunoglobulin sequences (see, e.g., WO 1998/24893; WO 1996/34096; WO 1996/33735; WO 1991/10741; Jakobovits et al., *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits et al., *Nature* 362: 255-258 (1993); Bruggemann et al., *Year in Immunol.* 7:33 (1993); U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; and 5,661,016; Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild et al., *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996); and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also, e.g., Vaswani and Hamilton, *Ann. Allergy, Asthma* & Immunol. 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994); and U.S. Pat. Nos. 6,982,321 and 7,087,409. See also van Dijk and van de Winkel, *Curr. Opin. Pharmacol.,* 5: 368-74 (2001). Human antibodies can be prepared by administering the antigen to a transgenic animal that has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, e.g., immunized xenomice (see, e.g., U.S. Pat. Nos. 6,075,181 and 6,150,584 regarding XENOMOUSE™ technology). See also, for example, Li et al., *Proc. Natl. Acad. Sci. USA,* 103:3557-3562 (2006) regarding human antibodies generated via a human B-cell hybridoma technology. The humanized antibody may also include a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art. In one embodiment, the human antibody is selected from a phage library, where that phage library expresses human antibodies (Vaughan et al. *Nature Biotechnology* 14:309-314 (1996): Sheets et al. *PNAS* (*USA*) 95:6157-6162 (1998)); Hoogenboom and Winter, *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581 (1991)). Human antibodies can also be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody rep-

ertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., *Bio/Technology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild et al., *Nature Biotechnology* 14: 845-51 (1996); Neuberger, *Nature Biotechnology* 14: 826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995). Alternatively, the human antibody may be prepared via immortalization of human B lymphocytes producing an antibody directed against a target antigen (such B lymphocytes may be recovered from an individual or may have been immunized in vitro). See, e.g., Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985); Boerner et al., *J. Immunol.,* 147 (1):86-95 (1991); and U.S. Pat. No. 5,750,373.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs/HVRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al., Bio/Technology 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR/HVR and/or framework residues is described by: Barbas et al., Proc Nat. Acad. Sci. USA 91:3809-3813 (1994); Schier et al., Gene 169: 147-155 (1995); Yelton et al., J. Immunol. 155:1994-2004 (1995); Jackson et al., J. Immunol. 154(7):3310-9 (1995); and Hawkins et al., J. Mol. Biol. 226:889-896 (1992).

The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "VH." The variable domain of the light chain may be referred to as "VL." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions (HVRs) both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a beta-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "hypervariable region," "HVR," or "HV," when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. For example, the term hypervariable region refers to the regions of an antibody variable domain which are hypervariable in sequence and/or form structurally defined loops. Generally, antibodies comprise six HVRs; three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). In native antibodies, H3 and L3 display the most diversity of the six HVRs, and H3 in particular is believed to play a unique role in conferring fine specificity to antibodies. See, e.g., Xu et al., *Immunity* 13:37-45 (2000); Johnson and Wu, in *Methods in Molecular Biology* 248:1-25 (Lo, ed., Human Press, Totowa, N.J., 2003). Indeed, naturally occurring camelid antibodies consisting of a heavy chain only are functional and stable in the absence of light chain. See, e.g., Hamers-Casterman et al., *Nature* 363:446-448 (1993); Sheriff et al., *Nature Struct. Biol.* 3:733-736 (1996).

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ϵ, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas et al., Cellular and Mol. Immunology, 4th ed. (2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

The term "Fc region" is used to define the C-terminal region of an immunoglobulin heavy chain which may be generated by papain digestion of an intact antibody. The Fc region may be a native sequence Fc region or a variant Fc region. The Fc region of an immunoglobulin generally comprises two constant domains, a CH2 domain and a CH3 domain, and optionally comprises a CH4 domain.

By "Fc region chain" herein is meant one of the two polypeptide chains of an Fc region.

The "CH2 domain" of a human IgG Fc region (also referred to as "Cg2" domain) is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. It has been speculated that the carbohydrate may provide a substitute for the domain-domain pairing and help stabilize the CH2 domain. Burton, *Molec. Immunol.* 22:161-206 (1985). The CH2 domain herein may be a native sequence CH2 domain or variant CH2 domain.

The "CH3 domain" comprises the stretch of residues C-terminal to a CH2 domain in an Fc region. The CH3 region herein may be a native sequence CH3 domain or a variant CH3 domain (e.g. a CH3 domain with an introduced "protroberance" in one chain thereof and a corresponding introduced "cavity" in the other chain thereof; see U.S. Pat. No. 5,821,333, expressly incorporated herein by reference). Such variant CH3 domains may be used to make multispecific (e.g. bispecific) antibodies as herein described.

"Hinge region" herein may be a native sequence hinge region or a variant hinge region. The two polypeptide chains of a variant hinge region generally retain at least one cysteine residue per polypeptide chain, so that the two polypeptide chains of the variant hinge region can form a

disulfide bond between the two chains. The preferred hinge region herein is a native sequence human hinge region, e.g. a native sequence human IgG1 hinge region.

A "functional Fc region" possesses at least one "effector function" of a native sequence Fc region. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (e.g. an antibody variable domain) and can be assessed using various assays known in the art for evaluating such antibody effector functions.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgG1 Fc region (non-A and A allotypes); native sequence human IgG2 Fc region; native sequence human IgG3 Fc region; and native sequence human IgG4 Fc region as well as naturally occurring variants thereof.

An "intact" antibody is one which comprises an antigen-binding variable region as well as a light chain constant domain (C_L) and heavy chain constant domains, C_H1, C_H2 and C_H3. The constant domains may be native sequence constant domains (e.g. human native sequence constant domains) or amino acid sequence variant thereof. Preferably, the intact antibody has one or more effector functions.

A "parent antibody" or "wild-type" antibody is an antibody comprising an amino acid sequence which lacks one or more amino acid sequence alterations compared to an antibody variant as herein disclosed. Thus, the parent antibody generally has at least one hypervariable region which differs in amino acid sequence from the amino acid sequence of the corresponding hypervariable region of an antibody variant as herein disclosed. The parent polypeptide may comprise a native sequence (i.e. a naturally occurring) antibody (including a naturally occurring allelic variant), or an antibody with pre-existing amino acid sequence modifications (such as insertions, deletions and/or other alterations) of a naturally occurring sequence. Throughout the disclosure, "wild type," "WT," "wt," and "parent" or "parental" antibody are used interchangeably.

As used herein, "antibody variant" or "variant antibody" refers to an antibody which has an amino acid sequence which differs from the amino acid sequence of a parent antibody. Preferably, the antibody variant comprises a heavy chain variable domain or a light chain variable domain having an amino acid sequence which is not found in nature. Such variants necessarily have less than 100% sequence identity or similarity with the parent antibody. In a preferred embodiment, the antibody variant will have an amino acid sequence from about 75% to less than 100% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the parent antibody, more preferably from about 80% to less than 100%, more preferably from about 85% to less than 100%, more preferably from about 90% to less than 100%, and most preferably from about 95% to less than 100%. The antibody variant is generally one which comprises one or more amino acid alterations in or adjacent to one or more hypervariable regions thereof.

A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification. In certain embodiments, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will typically possess, e.g., at least about 80% sequence identity with a native sequence Fc region and/or with an Fc region of a parent polypeptide, or at least about 90% sequence identity therewith, or at least about 95% sequence or more identity therewith.

Antibody "effector functions" refer to those biological activities attributable to the Fc region (a native sequence Fc region or amino acid sequence variant Fc region) of an antibody, and vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. Natural Killer (NK) cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in U.S. Pat. No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al. *PNAS* (*USA*) 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. In certain embodiments, the cells express at least FcγRIII and perform ADCC effector function(s). Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being generally preferred. The effector cells may be isolated from a native source thereof, e.g. from blood or PBMCs as described herein.

"Fc receptor" or "FcR" describes a receptor that binds to the Fc region of an antibody. In some embodiments, an FcR is a native human FcR. In some embodiments, an FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of those receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cyto-

plasmic domain. (see, e.g., Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed, for example, in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein.

The term "Fc receptor" or "FcR" also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., *J. Immunol.* 117:587 (1976) and Kim et al., *J. Immunol.* 24:249 (1994)) and regulation of homeostasis of immunoglobulins. Methods of measuring binding to FcRn are known (see, e.g., Ghetie and Ward., *Immunol. Today* 18(12):592-598 (1997); Ghetie et al., *Nature Biotechnology,* 15(7):637-640 (1997); Hinton et al., *J. Biol. Chem.* 279(8):6213-6216 (2004); WO 2004/92219 (Hinton et al.).

Binding to human FcRn in vivo and serum half life of human FcRn high affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates to which the polypeptides with a variant Fc region are administered. WO 2000/42072 (Presta) describes antibody variants with improved or diminished binding to FcRs. See also, e.g., Shields et al. *J. Biol. Chem.* 9(2):6591-6604 (2001).

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass), which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g., as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed. Polypeptide variants with altered Fc region amino acid sequences (polypeptides with a variant Fc region) and increased or decreased C1q binding capability are described, e.g., in U.S. Pat. No. 6,194,551 B1 and WO 1999/51642. See also, e.g., Idusogie et al. *J. Immunol.* 164: 4178-4184 (2000).

An "affinity matured" antibody is one with one or more alterations in one or more CDRs thereof which result an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). In one embodiment, an affinity matured antibody has nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al. *Bio/Technology* 10:779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by: Barbas et al. *Proc Nat. Acad. Sci, USA* 91:3809-3813 (1994); Schier et al. *Gene* 169:147-155 (1995); Yelton et al. *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al, *J. Mol. Biol.* 226:889-896 (1992).

The term "therapeutic antibody" refers to an antibody that is used in the treatment of disease. A therapeutic antibody may have various mechanisms of action. A therapeutic antibody may bind and neutralize the normal function of a target associated with an antigen. For example, a monoclonal antibody that blocks the activity of the of protein needed for the survival of a cancer cell causes the cell's death. Another therapeutic monoclonal antibody may bind and activate the normal function of a target associated with an antigen. For example, a monoclonal antibody can bind to a protein on a cell and trigger an apoptosis signal. Yet another monoclonal antibody may bind to a target antigen expressed only on diseased tissue; conjugation of a toxic payload (effective agent), such as a chemotherapeutic or radioactive agent, to the monoclonal antibody can create an agent for specific delivery of the toxic payload to the diseased tissue, reducing harm to healthy tissue. A "biologically functional fragment" of a therapeutic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

The antibody may bind to any protein, including, without limitation, a member of the HER receptor family, such as HER1 (EGFR), HER2, HER3 and HER4; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD21, CD22, and CD34; cell adhesion molecules such as LFA-1, Mol, p150,95, VLA-4, ICAM-1, VCAM and av/p3 integrin including either α or β or subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as vascular endothelial growth factor (VEGF); IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; and protein C. Other exemplary proteins include growth hormone (GH), including human growth hormone (hGH) and bovine growth hormone (bGH); growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA); bombazine; thrombin; tumor necrosis factor-α and -β; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); serum albumin such as human serum albumin (HSA); mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins (IGFBPs); erythropoietin (EPO); thrombopoietin (TPO); osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and biologically active fragments or variants of any of the above-listed polypeptides. Many other antibodies and/or other proteins may be used in accordance with the instant invention, and the above lists are not meant to be limiting.

Therapeutic antibodies of particular interest include those in clinical oncological practice or development such as commercially available AVASTIN® (bevacizumab), HERCEPTIN® (trastuzumab), LUCENTIS® (ranibizumab), RAPTIVA® (efalizumab), RITUXAN® (rituximab), and XOLAIR® (omalizumab), as well as, anti-amyloid beta

(Abeta), anti-CD4 (MTRX1011A), anti-EGFL7 (EGF-like-domain 7), anti-IL13, Apomab (anti-DR5-targeted pro-apoptotic receptor agonist (PARA), anti-BR3 (CD268, BLyS receptor 3, BAFF-R, BAFF Receptor), anti-beta 7 integrin subunit, dacetuzumab (Anti-CD40), GA101 (anti-CD20 monoclonal antibody), MetMAb (anti-MET receptor tyrosine kinase), anti-neuropilin-1 (NRP1), ocrelizumab (anti-CD20 antibody), anti-OX40 ligand, anti-oxidized LDL (ox-LDL), pertuzumab (HER dimerization inhibitors (HDIs), and. rhuMAb IFN alpha.

A "biologically functional fragment" of an antibody comprises only a portion of an intact antibody, wherein the portion retains at least one, and as many as most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, a biologically functional fragment of an antibody comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, a biologically functional fragment of an antibody, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, a biologically functional fragment of an antibody is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such a biologically functional fragment of an antibody may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

The term "diagnostic protein" refers to a protein that is used in the diagnosis of a disease.

The term "diagnostic antibody" refers to an antibody that is used as a diagnostic reagent for a disease. The diagnostic antibody may bind to a target antigen that is specifically associated with, or shows increased expression in, a particular disease. The diagnostic antibody may be used, for example, to detect a target in a biological sample from a patient, or in diagnostic imaging of disease sites, such as tumors, in a patient. A "biologically functional fragment" of a diagnostic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

"Purified" means that a molecule is present in a sample at a concentration of at least 80-90% by weight of the sample in which it is contained. The protein, including antibodies, which is purified is preferably essentially pure and desirably essentially homogeneous (i.e. free from contaminating proteins etc.).

An "essentially pure" protein means a protein composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight.

An "essentially homogeneous" protein means a protein composition comprising at least about 99% by weight of protein, based on total weight of the composition.

As used herein, "soluble" refers to polypeptides that, when in aqueous solutions, are completely dissolved, resulting in a clear to slightly opalescent solution with no visible particulates, as assessed by visual inspection. A further assay of the turbidity of the solution (or solubility of the protein) may be made by measuring UV absorbances at 340 nm to 360 nm with a 1 cm path-length cell where turbidity at 20 mg/ml is less than 0.05 absorbance units.

An "isolated" antibody or polypeptide is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater than 99% by weight; (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The terms "Protein A" and "ProA" are used interchangeably herein and encompasses Protein A recovered from a native source thereof, Protein A produced synthetically (e.g. by peptide synthesis or by recombinant techniques), and variants thereof which retain the ability to bind proteins which have a C_H2/C_H3 region, such as an Fc region. Protein A can be purchased commercially from Repligen, Pharmacia and Fermatech. Protein A is generally immobilized on a solid phase support material. The term "ProA" also refers to an affinity chromatography resin or column containing chromatographic solid support matrix to which is covalently attached Protein A.

The term "chromatography" refers to the process by which a solute of interest in a mixture is separated from other solutes in a mixture as a result of differences in rates at which the individual solutes of the mixture migrate through a stationary medium under the influence of a moving phase, or in bind and elute processes.

The term "affinity chromatography" and "protein affinity chromatography" are used interchangeably herein and refer to a protein separation technique in which a protein of interest or antibody of interest is reversibly and specifically bound to a biospecific ligand. Preferably, the biospecific ligand is covalently attached to a chromatographic solid phase material and is accessible to the protein of interest in solution as the solution contacts the chromatographic solid phase material. The protein of interest (e.g., antibody, enzyme, or receptor protein) retains its specific binding affinity for the biospecific ligand (antigen, substrate, cofactor, or hormone, for example) during the chromatographic steps, while other solutes and/or proteins in the mixture do not bind appreciably or specifically to the ligand. Binding of the protein of interest to the immobilized ligand allows contaminating proteins or protein impurities to be passed through the chromatographic medium while the protein of interest remains specifically bound to the immobilized ligand on the solid phase material. The specifically bound protein of interest is then removed in active form from the immobilized ligand with low pH, high pH, high salt, competing ligand, and the like, and passed through the chromatographic column with the elution buffer, free of the contaminating proteins or protein impurities that were earlier allowed to pass through the column. Any component can be used as a ligand for purifying its respective specific binding protein, e.g. antibody.

The terms "non-affinity chromatography" and "non-affinity purification" refer to a purification process in which affinity chromatography is not utilized. Non-affinity chromatography includes chromatographic techniques that rely

on non-specific interactions between a molecule of interest (such as a protein, e.g. antibody) and a solid phase matrix.

A "cation exchange resin" refers to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin may, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-methyl-cellulose, sulphopropyl (SP) immobilized on agarose (e.g. SP-SEPHAROSE FAST FLOW™ or SP-SEPHAROSE HIGH PERFORMANCE™, from Pharmacia) and sulphonyl immobilized on agarose (e.g. S-SEPHAROSE FAST FLOW™ from Pharmacia). A "mixed mode ion exchange resin" refers to a solid phase which is covalently modified with cationic, anionic, and hydrophobic moieties. A commercially available mixed mode ion exchange resin is BAKERBOND ABX™ (J. T. Baker, Phillipsburg, N.J.) containing weak cation exchange groups, a low concentration of anion exchange groups, and hydrophobic ligands attached to a silica gel solid phase support matrix.

The term "anion exchange resin" is used herein to refer to a solid phase which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Commercially available anion exchange resins include DEAE cellulose, QAE SEPHADEX™ and FAST Q SEPHAROSE™ (Pharmacia).

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in *Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., ed. Calbiochem Corporation (1975). In one embodiment, the buffer has a pH in the range from about 2 to about 9, alternatively from about 3 to about 8, alternatively from about 4 to about 7 alternatively from about 5 to about 7. Non-limiting examples of buffers that will control the pH in this range include MES, MOPS, MOPSO, Tris, HEPES, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

The "loading buffer" is that which is used to load the composition comprising the polypeptide molecule of interest and one or more impurities onto the ion exchange resin. The loading buffer has a conductivity and/or pH such that the polypeptide molecule of interest (and generally one or more impurities) is/are bound to the ion exchange resin or such that the protein of interest flows through the column while the impurities bind to the resin.

The "intermediate buffer" is used to elute one or more impurities from the ion exchange resin, prior to eluting the polypeptide molecule of interest. The conductivity and/or pH of the intermediate buffer is/are such that one or more impurity is eluted from the ion exchange resin, but not significant amounts of the polypeptide of interest.

The term "wash buffer" when used herein refers to a buffer used to wash or re-equilibrate the ion exchange resin, prior to eluting the polypeptide molecule of interest. Conveniently, the wash buffer and loading buffer may be the same, but this is not required.

The "elution buffer" is used to elute the polypeptide of interest from the solid phase. The conductivity and/or pH of the elution buffer is/are such that the polypeptide of interest is eluted from the ion exchange resin.

A "regeneration buffer" may be used to regenerate the ion exchange resin such that it can be re-used. The regeneration buffer has a conductivity and/or pH as required to remove substantially all impurities and the polypeptide of interest from the ion exchange resin.

The term "substantially similar" or "substantially the same," as used herein, denotes a sufficiently high degree of similarity between two numeric values (for example, one associated with an antibody of the invention and the other associated with a reference/comparator antibody), such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, and/or less than about 10% as a function of the reference/comparator value.

The phrase "substantially reduced," or "substantially different," as used herein with regard to amounts or numerical values (and not as reference to the chemical process of reduction), denotes a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, and/or greater than about 50% as a function of the value for the reference/comparator molecule.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors," or simply, "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector.

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled

in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, Calif., or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B.

It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

"Percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a reference Factor D-encoding sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. Sequence identity is then calculated relative to the longer sequence, i.e. even if a shorter sequence shows 100% sequence identity with a portion of a longer sequence, the overall sequence identity will be less than 100%.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. "Treatment" herein encompasses alleviation of the disease and of the signs and symptoms of the particular disease.

A "disorder" is any condition that would benefit from treatment with the protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include carcinomas and allergies.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, non-human higher primates, other vertebrates, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

B. Exemplary Methods and Materials for Carrying Out the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology and the like, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., *Molecular Cloning: A Laboratory Manual*, (J. Sambrook et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989); *Current Protocols in Molecular Biology* (F. Ausubel et al., eds., 1987 updated); *Essential Molecular Biology* (T. Brown ed., IRL Press 1991); *Gene Expression Technology* (Goeddel ed., Academic Press 1991); *Methods for Cloning and Analysis of Eukaryotic Genes* (A. Bothwell et al., eds., Bartlett Publ. 1990); *Gene Transfer and Expression* (M. Kriegler, Stockton Press 1990); *Recombinant DNA Methodology II* (R. Wu et al., eds., Academic Press 1995); PCR: *A Practical Approach* (M. McPherson et al., IRL Press at Oxford University Press 1991); *Oligonucleotide Synthesis* (M. Gait ed., 1984); *Cell Culture for Biochemists* (R. Adams ed., Elsevier Science Publishers 1990); *Gene Transfer Vectors for Mammalian Cells* (J. Miller & M. Calos eds., 1987); *Mammalian Cell Biotechnology* (M. Butler ed., 1991); *Animal Cell Culture* (J. Pollard et al., eds., Humana Press 1990); *Culture of Animal Cells*, 2nd Ed. (R. Freshney et al., eds., Alan R. Liss 1987); *Flow Cytometry and Sorting* (M. Melamed et al., eds., Wiley-Liss 1990); the series *Methods in Enzymology* (Academic Press, Inc.); Wirth M. and Hauser H. (1993); *Immunochemistry in Practice*, 3rd edition, A. Johnstone & R. Thorpe, Blackwell Science, Cambridge, Mass., 1996; *Techniques in Immunocytochemistry*, (G. Bullock & P. Petrusz eds., Academic Press 1982, 1983, 1985, 1989); Handbook of Experimental Immunology, (D. Weir & C. Blackwell, eds.); *Current Protocols in Immunology* (J. Coligan et al., eds. 1991); *Immunoassay* (E. P. Diamandis & T. K. Christopoulos, eds., Academic Press, Inc., 1996); Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed) Academic Press, New York; Ed Harlow and David Lane, *Antibodies A laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988; *Antibody Engineering*, 2nd edition (C. Borrebaeck, ed., Oxford University Press, 1995); and the series *Annual Review of Immunology*; the series *Advances in Immunology*.

1. Recombinant Production of Proteins in Mammalian Host Cells Using a Glutamine Free Cell Culture Medium

The present invention concerns the large-scale recombinant production of proteins in mammalian host cells, using a glutamine-free cell culture medium supplemented with asparagine. Mammalian cells have become the dominant system for the production of mammalian proteins for clinical applications, primarily due to their ability to produce properly folded and assembled heterologous proteins, and their capacity for post-translational modifications. Chinese hamster ovary (CHO) cells, and cell lines obtained from various other mammalian sources, such as, for example, mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) and human retinal cells have been approved by regulatory agencies for the production of

biopharmaceutical products, including therapeutic antibodies. Of these, Chinese Hamster Ovary Cells (CHO) are among the most commonly used industrial hosts, which are widely employed for the production of heterologous proteins. Thus, methods for the large-scale production of antibodies in CHO, including dihydrofolate reductase negative (DHFR−) CHO cells, are well known in the art (see, e.g. Trill et al., *Curr. Opin. Biotechnol.* 6(5):553-60 (1995) and U.S. Pat. No. 6,610,516).

As a first step, the nucleic acid (e.g., cDNA or genomic DNA) encoding the desired recombinant protein may be inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below. Optional signal sequences, origins of replication, marker genes, enhancer elements and transcription terminator sequences that may be employed are known in the art and described in further detail in PCT Publication WO 97/25428.

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the protein-encoding nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding the desired protein by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector.

Promoters suitable for use with prokaryotic and eukaryotic hosts are known in the art, and are described in further detail in PCT Publication No. WO97/25428.

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* cells, such as *E. coli* K12 strain 294 (ATCC® 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced using standard techniques known in the art. (See, e.g., Messing et al., *Nucleic Acids Res.* 1981, 9:309; Maxam et al., Methods in Enzymology 1980, 65:499).

Expression vectors that provide for the transient expression in mammalian cells may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector (Sambrook et al., supra). Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of a desired heterologous protein in recombinant vertebrate cell culture are described in Gething et al., *Nature* 1981, 293:620-625; Mantei et al., *Nature* 1979, 281:40-46; EP 117,060; and EP 117,058.

For large-scale production, according to the present invention mammalian host cells are transfected and preferably transformed with the above-described expression vectors and cultured in nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described (Shaw et al., Gene 1983, 23:315 and PCT Publication No. WO 89/05859). In addition, plants may be transfected using ultrasound treatment, PCT Publication No. WO 91/00358 published 10 Jan. 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method (Graham and van der Eb, *Virology* 1978, 52:456-457) may be employed. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. For various techniques for transforming mammalian cells, see also Keown et al. *Methods in Enzymology* 1990, 185:527-537 and Mansour et al. *Nature* 1988, 336:348-352.

During large-scale production, to begin the production cycle usually a small number of transformed recombinant host cells is allowed to grow in culture for several days. Once the cells have undergone several rounds of replication, they are transferred to a larger container where they are prepared to undergo fermentation. The media in which the cells are grown and the levels of oxygen, nitrogen and carbon dioxide that exist during the production cycle may have a significant impact on the production process. Growth parameters are determined specifically for each cell line and these parameters are measured frequently to assure optimal growth and production conditions.

When the cells grow to sufficient numbers, they are transferred to large-scale production tanks to begin the production phase, and grown for a longer period of time. At this point in the process, the recombinant protein can be harvested. Typically, the cells are engineered to secrete the polypeptide into the cell culture media, so the first step in the purification process is to separate the cells from the media. Harvesting usually includes centrifugation and filtration to produce a Harvested Cell Culture Fluid (HCCF). The media is then subjected to several additional purification steps that remove any cellular debris, unwanted proteins, salts, minerals or other undesirable elements. At the end of the

purification process, the recombinant protein is highly pure and is suitable for human therapeutic use.

Although this process has been the subject of much study and improvements over the past several decades, there is room fur further improvements in the large-scale commercial production of recombinant proteins, such as antibodies. Thus, increases in cell viability, longevity and specific productivity of mammalian host cell cultures, and improvements in the titer of the recombinant proteins produced have a genuine impact on the price of the recombinant protein produced, and, in the case of therapeutic proteins, the price and availability of drug products.

The present invention concerns an improved method for the production of heterologous proteins in mammalian cell culture, using a glutamine-free culture medium with added asparagine in the production phase of the cell culture process. The culture media used in the process of the present invention can be based on any commercially available medium for recombinant production of proteins in mammalian host cells, in particular CHO cells.

Examples of commercially available culture media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. In addition, the culture media of the present invention can be based any of the media described in Ham and McKeehan, Meth. Enz., 58: 44 (1979); Barnes and Sato, Anal. Biochem., 102: 255 (1980); U.S. Pat. No. 4,767,704; U.S. Pat. No. 4,657,866; U.S. Pat. No. 4,927,762; U.S. Pat. No. 5,122,469 or U.S. Pat. No. 4,560,655; WO 90/03430; and WO 87/00195, provided that glutamine is omitted as an ingredient.

Under Glutamine-free conditions Asparagine is required since mammalian cells can synthesize Asparagine only in presence of Glutamine. Asparagine is synthesized by amide transfer from Glutamine in the presence of Asparagine synthetase. The Asparagine is preferably added to the culture medium at a concentration in the range of 2.5 mM to 15 mM. In various embodiments of the present invention, the preferred concentration of Asparagine should be at least 2.5 mM. In preferred embodiments, the asparagine is added at a concentration of 10 mM.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in, and can be adapted for the production of recombinant proteins using the cell culture media herein.

The necessary nutrients and growth factors for the medium, including their concentrations, for a particular cell line, are determined empirically without undue experimentation as described, for example, in *Mammalian Cell Culture*, Mather, ed. (Plenum Press: NY, 1984); Barnes and Sato, *Cell,* 22: 649 (1980) or *Mammalian Cell Biotechnology: A Practical Approach*, M. Butler, ed. (IRL Press, 1991).

A suitable medium contains a basal medium component such as a DMEM/HAM F-12-based formulation (for composition of DMEM and HAM F12 media and especially serum-free media, see culture media formulations in American Type Culture Collection Catalogue of Cell Lines and Hybridomas, Sixth Edition, 1988, pages 346-349), with modified concentrations of some components such as amino acids, salts, sugar, and vitamins, and optionally containing glycine, hypoxanthine, and thymidine; recombinant human insulin, hydrolyzed peptone, such as PRIMATONE HS™ or PRIMATONE RL™ (Sheffield, England), or the equivalent; a cell protective agent, such as PLURONIC F68™ or the equivalent pluronic polyol; GENTAMYCIN™; and trace elements. The formulations of medium as described in U.S. Pat. No. 5,122,469, characterized by the presence of high levels of certain amino acids, as well as PS-20 as described below, are particularly appropriate.

The glycoproteins of the present invention may be produced by growing cells which express the desired glycoprotein under a variety of cell culture conditions. For instance, cell culture procedures for the large- or small-scale production of glycoproteins are potentially useful within the context of the present invention. Procedures including, but not limited to, a fluidized bed bioreactor, hollow fiber bioreactor, roller bottle culture, or stirred tank bioreactor system may be used, in the later two systems, with or without microcarriers, and operated alternatively in a batch, fed-batch, or continuous mode.

In a particular embodiment the cell culture of the present invention is performed in a stirred tank bioreactor system and a fed-batch culture procedure is employed. In the preferred fed-batch culture the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed-batch culture can include, for example, a semi-continuous fed-batch culture, wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium Fed-batch culture is distinguished from simple-batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed-batch culture can be further distinguished from perfusion culturing insofar as the supernate is not removed from the culturing vessel during the process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers, etc., and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cells of the culture may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, the present invention contemplates a single-step or multiple-step culture procedure. In a single-step culture the host cells are inoculated into a culture environment and the processes of the instant invention are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture is envisioned. In the multi-stage culture cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

According to a specific aspect of the invention, fed-batch or continuous cell culture conditions are devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In the growth phase cells are grown under conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (DO_2), and the like, are those used with the particular host and will be apparent to the ordinarily-skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO_2) or a base (e.g., Na_2CO_3 or NaOH). A suitable temperature range for culturing mammalian cells such as CHO cells is between about 30 to 40° C. and preferably about 37° C. and a suitable DO_2 is between 5-90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above the production phase or step may be continuous with the inoculation or growth phase or step.

Production of a target protein in mammalian, e.g., CHO, cells typically employs a semi-continuous process whereby cells are culture in a "seed-train" for various periods of time and are periodically transferred to inoculum fermentors to generate enough cell mass to inoculate a production fermentor at larger scale. Thus, cells used for the production of the desired protein are in culture for various periods of time up to a maximum predefined cell age. The parameters of the cell culture process, such as seed density, pH, DO_2 and temperature during culture, duration of the production culture, operating conditions of harvest, etc. are a function of the particular cell line and culture medium used, and can be determined empirically, without undue experimentation.

According to the present invention, the cell-culture environment during the production phase of the cell culture is controlled. In a preferred aspect, the production phase of the cell culture process is preceded by a transition phase of the cell culture in which parameters for the production phase of the cell culture are engaged.

The desired polypeptide, such as antibody, preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly produced without a secretory signal. If the polypeptide is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g., Triton-X 100) or its extracellular region may be released by enzymatic cleavage.

When the polypeptide is produced in a recombinant cell other than one of human origin, it is free of proteins or polypeptides of human origin. However, it is usually necessary to recover or purify recombinant proteins from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the desired polypeptide. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. The heterologous polypeptide thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column such as SP-Sepharose™ or CM-Sepharose™; hydroxyapatite; hydrophobic interaction chromatography; ethanol precipitation; chromatofocusing; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G75™; and/or diafiltration.

Recombinant polypeptides can be isolated, e.g. by affinity chromatography.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for the purification and isolation of recombinant proteins, including antibodies, can be used herein, and modified if needed, using standard techniques.

Expression of the desired heterologous protein may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA* 1980, 77:5201-5205), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal.

2. Antibodies

In a preferred embodiment, the methods of the present invention are used for the recombinant production of antibodies, including therapeutic and diagnostic antibodies. Antibodies within the scope of the present invention include, but are not limited to: anti-HER2 antibodies including Trastuzumab (HERCEPTIN®) (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285-4289 (1992), U.S. Pat. No. 5,725,856); anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN®), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108B1, or Tositumomab (BEXXAR®); anti-IL-8 (St John et al., *Chest,* 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors,* 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., *Transplant Intl.* 4:3-7 (1991), and Hourmant et al., *Transplantation* 58:377-380 (1994)); anti-IgE (Presta et al., *J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul. 31, 1997); anti-IgE (including E25,

E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No. 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No. PCT/US98/13410 filed Jun. 30, 1998, U.S. Pat. No. 5,714,338); anti-Apo-2 receptor antibody (WO 98/51793 published Nov. 19, 1998); anti-TNF-α antibodies including cA2 (REMICADE®), CDP571 and MAK-195 (See, U.S. Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al., *J Immunol.* 156(4):1646-1653 (1996), and Dhainaut et al., *Crit. Care Med.* 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human $\alpha_4\beta_7$ integrin (WO 98/06248 published Feb. 19, 1998); anti-EGFR (chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S. Pat. No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-tac antibodies such as CHI-621 (SIMULECT®) and (ZENAPAX®) (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997); anti-CD4 antibodies such as the cM-7412 antibody (Choy et al., *Arthritis Rheum* 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al., *Nature* 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22 antibody directed against FcγRI as in Graziano et al., *J. Immunol.* 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al., *Cancer Res.* 55(23Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al., *Cancer Res.* 55(23): 5852s-5856s (1995); and Richman et al., *Cancer Res.* 55(23 Supp): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al., *Eur J. Immunol.* 26(1):1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al., *J. Immunol.* 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al., *Cancer Res* 55(23 Suppl):5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22 antibodies such as LL2 or LymphoCide (Juweid et al., *Cancer Res* 55(23 Suppl):5899s-5907s (1995)); anti-EpCAM antibodies such as 17-1A (PANOREX®); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO®); anti-RSV antibodies such as MEDI-493 (SYNAGIS®); anti-CMV antibodies such as PROTOVIR®; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR®; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-αvβ3 antibody VITAXIN®; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1). The preferred target antigens for the antibody herein are: HER2 receptor, VEGF, IgE, CD20, CD11a, and CD40.

Many of these antibodies are widely used in clinical practice to treat various diseases, including cancer.

In certain specific embodiments, the methods of the present invention are used for the production of the following antibodies and recombinant proteins.

Anti-CD20 Antibodies

Rituximab (RITUXAN®) is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. Rituximab is the antibody called "C2B8" in U.S. Pat. No. 5,736,137 issued Apr. 7, 1998 (Anderson et al.). Rituximab is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20-positive, B cell non-Hodgkin's lymphoma. In vitro mechanism of action studies have demonstrated that rituximab binds human complement and lyses lymphoid B cell lines through complement-dependent cytotoxicity (CDC) (Reff et al., *Blood* 83(2):435-445 (1994)). Additionally, it has significant activity in assays for antibody-dependent cellular cytotoxicity (ADCC). More recently, rituximab has been shown to have anti-proliferative effects in tritiated thymidine incorporation assays and to induce apoptosis directly, while other anti-CD19 and CD20 antibodies do not (Maloney et al., *Blood* 88(10):637a (1996)). Synergy between rituximab and chemotherapies and toxins has also been observed experimentally. In particular, rituximab. sensitizes drug-resistant human B cell lymphoma cell lines to the cytotoxic effects of doxorubicin, CDDP, VP-1 6, diphtheria toxin and ricin (Demidem et al., *Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997)). In vivo preclinical studies have shown that rituximab depletes B cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff et al., *Blood* 83(2):435-445 (1994)).

Patents and patent publications concerning CD20 antibodies include U.S. Pat. Nos. 5,776,456, 5,736,137, 6,399,061, and 5,843,439, as well as U.S. patent application Nos. US 2002/0197255A1, US 2003/0021781A1, US 2003/0082172 A1, US 2003/0095963 A1, US 2003/0147885 A1 (Anderson et al.); U.S. Pat. No. 6,455,043B1 and WO00/09160 (Grillo-Lopez, A.); WO00/27428 (Grillo-Lopez and White); WO00/27433 (Grillo-Lopez and Leonard); WO00/44788 (Braslawsky et al.); WO01/10462 (Rastetter, W.); WO01/10461 (Rastetter and White); WO01/10460 (White and Grillo-Lopez); U.S. application No. US2002/0006404 and WO02/04021 (Hanna and Hariharan); U.S. application No. US2002/0012665 A1 and WO01/74388 (Hanna, N.); U.S. application No. US 2002/0058029 A1 (Hanna, N.); U.S. application No. US 2003/0103971 A1 (Hariharan and Hanna); U.S. application No. US2002/0009444A1, and WO01/80884 (Grillo-Lopez, A.); WO01/97858 (White, C.); U.S. application No. US2002/0128488A1 and WO02/34790 (Reff, M.); W02/060955 (Braslawsky et al.); WO2/096948 (Braslawsky et al.); WO02/079255 (Reff and Davies); U.S. Pat. No. 6,171,586B1, and WO98/56418 (Lam et al.); WO98/58964 (Raju, S.); WO99/22764 (Raju, S.); WO99/51642, U.S. Pat. No. 6,194,551B1, U.S. Pat. No. 6,242,195B1, U.S. Pat. No. 6,528,624B1 and U.S. Pat. No. 6,538,124 (Idusogie et al.); WO00/42072 (Presta, L.); WO00/67796 (Curd et al.); WO01/03734 (Grillo-Lopez et al.); U.S. application No. US 2002/0004587A1 and WO01/77342 (Miller and Presta); U.S. application No. US2002/0197256 (Grewal, I.); U.S. application No. US 2003/0157108 A1 (Presta, L.); U.S. Pat. Nos. 6,090,365B1, 6,287,537B1, 6,015,542, 5,843,398, and 5,595,721, (Kaminski et al.); U.S. Pat. Nos. 5,500,362, 5,677,180, 5,721,108, and 6,120,767 (Robinson et al.); U.S. Pat. No. 6,410,391B1 (Raubitschek et al.); U.S. Pat. No. 6,224,866B1 and WO00/20864 (Barbera-Guillem, E.); WO01/13945 (Barbera-Guillem, E.); WO00/67795 (Goldenberg); U.S. application No. US 2003/01339301 A1 and WO00/74718 (Goldenberg and Hansen); WO00/76542 (Golay et al.); WO01/72333 (Wolin and Rosenblatt); U.S. Pat. No. 6,368,596B1 (Ghetie et al.); U.S. application No. US2002/0041847 A1, (Goldenberg, D.); U.S. application No. US2003/0026801A1 (Weiner and Hartmann); WO02/102312 (Engleman, E.); U.S. patent application No. 2003/0068664 (Albitar et al.); WO03/002607 (Leung, S.); WO 03/049694 and US 2003/0185796 A1 (Wolin et al.); WO03/061694 (Sing and Siegall); US 2003/0219818 A1 (Bohen et al.); US 2003/0219433 A1 and WO 03/068821

(Hansen et al.) each of which is expressly incorporated herein by reference. See, also, U.S. Pat. No. 5,849,898 and EP application no. 330,191 (Seed et al.); U.S. Pat. No. 4,861,579 and EP332,865A2 (Meyer and Weiss); U.S. Pat. No. 4,861,579 (Meyer et al.) and WO95/03770 (Bhat et al.).

Publications concerning therapy with Rituximab include: Perotta and Abuel "Response of chronic relapsing ITP of 10 years duration to Rituximab" Abstract #3360 *Blood* 10(1) (part 1-2): p. 88B (1998); Stashi et al., "Rituximab chimeric anti-CD20 monoclonal antibody treatment for adults with chronic idopathic thrombocytopenic purpura" *Blood* 98(4): 952-957 (2001); Matthews, R. "Medical Heretics" New Scientist (7 Apr. 2001); Leandro et al., "Clinical outcome in 22 patients with rheumatoid arthritis treated with B lymphocyte depletion" *Ann Rheum Dis* 61:833-888 (2002); Leandro et al., "Lymphocyte depletion in rheumatoid arthritis: early evidence for safety, efficacy and dose response. *Arthritis & Rheumatism* 44(9): S370 (2001); Leandro et al., "An open study of B lymphocyte depletion in systemic lupus erythematosus", *Arthritis & Rheumatism* 46(1):2673-2677 (2002); Edwards and Cambridge "Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes" *Rheumatology* 40:205-211 (2001); Edwards et al., "B-lymphocyte depletion therapy in rheumatoid arthritis and other autoimmune disorders" *Biochem. Soc. Trans.* 30(4):824-828 (2002); Edwards et al., "Efficacy and safety of Rituximab, a B-cell targeted chimeric monoclonal antibody: A randomized, placebo controlled trial in patients with rheumatoid arthritis. *Arthritis & Rheumatism* 46(9): 5197 (2002); Levine and Pestronk "IgM antibody-related polyneuropathies: B-cell depletion chemotherapy using Rituximab" *Neurology* 52: 1701-1704 (1999); DeVita et al., "Efficacy of selective B cell blockade in the treatment of rheumatoid arthritis" *Arthritis & Rheumatism* 46:2029-2033 (2002); Hidashida et al., "Treatment of DMARD-Refractory rheumatoid arthritis with rituximab." Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002; Tuscano, J. "Successful treatment of Infliximab-refractory rheumatoid arthritis with rituximab" Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002. Sarwal et al., *N. Eng. J. Med.* 349(2):125-138 (Jul. 10, 2003) reports molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling.

In various embodiments, the invention provides pharmaceutical compositions comprising humanized anti-CD20 antibodies. In certain embodiments, the humanized antibody composition of the invention further comprises amino acid alterations in the IgG Fc and exhibits increased binding affinity for human FcRn over an antibody having wild-type IgG Fc, by at least 60 fold, at least 70 fold, at least 80 fold, more preferably at least 100 fold, preferably at least 125 fold, even more preferably at least 150 fold to about 170 fold.

The N-glycosylation site in IgG is at Asn297 in the C_H2 domain. Humanized antibody compositions of the present invention include compositions of any of the preceding humanized antibodies having an Fc region, wherein about 80-100% (and preferably about 90-99%) of the antibody in the composition comprises a mature core carbohydrate structure which lacks fucose, attached to the Fc region of the glycoprotein. Such compositions were demonstrated herein to exhibit a surprising improvement in binding to Fc(RIIIA (F158), which is not as effective as Fc(RIIIA (V158) in interacting with human IgG. Fc(RIIIA (F158) is more common than Fc(RIIIA (V158) in normal, healthy African Americans and Caucasians. See Lehrnbecher et al., *Blood* 94:4220 (1999). Historically, antibodies produced in Chinese Hamster Ovary Cells (CHO), one of the most commonly used industrial hosts, contain about 2 to 6% in the population that are nonfucosylated. YB2/0 and Lec13, however, can produce antibodies with 78 to 98% nonfucosylated species. Shinkawa et al., *J. Bio. Chem.* 278 (5), 3466-347 (2003), reported that antibodies produced in YB2/0 and Lec13 cells, which have less FUT8 activity, show significantly increased ADCC activity in vitro. The production of antibodies with reduced fucose content are also described in e.g., Li et al., (GlycoFi) "Optimization of humanized IgGs in glycoengineered *Pichia pastoris*" in Nature Biology online publication 22 Jan. 2006; Niwa R. et al., *Cancer Res.* 64(6):2127-2133 (2004); US 2003/0157108 (Presta); U.S. Pat. No. 6,602,684 and US 2003/0175884 (Glycart Biotechnology); US 2004/0093621, US 2004/0110704, US 2004/0132140 (all of Kyowa Hakko Kogyo).

A bispecific humanized antibody encompasses an antibody wherein one arm of the antibody has at least the antigen binding region of the H and/or L chain of a humanized antibody of the invention, and the other arm has V region binding specificity for a second antigen. In specific embodiments, the antigens are selected from the group consisting of CD-20, CD3, CD64, CD32A, CD16, NKG2D or other NK activating ligands.

Anti-HER2 Antibodies

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, trastuzumab or HERCEPTIN®; U.S. Pat. No. 5,821,337) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). Trastuzumab received marketing approval from the Food and Drug Administration (FDA) Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein. In November 2006, the FDA approved Herceptin as part of a treatment regimen containing doxorubicin, cyclophosphamide and paclitaxel, for the adjuvant treatment of patients with HER2-positive, node-positive breast cancer.

In various embodiments, the invention provides pharmaceutical compositions comprising humanized anti-HER2 antibodies. HER2 antibodies with various properties have been described in Tagliabue et al., *Int. J. Cancer* 47:933-937 (1991); McKenzie et al., *Oncogene* 4:543-548 (1989); Maier et al., *Cancer Res.* 51:5361-5369 (1991); Bacus et al., *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al., *PNAS* (*USA*) 88:8691-8695 (1991); Bacus et al., *Cancer Research* 52:2580-2589 (1992); Xu et al., *Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk et al., *Cancer Research* 52:2771-2776 (1992); Hancock et al., *Cancer Res.* 51:4575-4580 (1991); Shawver et al., *Cancer Res.* 54:1367-1373 (1994); Arteaga et al., *Cancer Res.* 54:3758-3765 (1994); Harwerth et al., *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; and Klapper et al., *Oncogene* 14:2099-2109 (1997).

Anti-VEGF Antibodies

anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors,* 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998) are FDA approved for the treatment of cancer. In various embodiments, the invention provides pharmaceutical compositions comprising humanized anti-VEGF antibodies.

Anti-CD11a Antibodies

The humanized anti-CD11a antibody efalizumab or Raptiva® (U.S. Pat. No. 6,037,454) received marketing approval from the Food and Drug Administration on Oct. 27, 2003 for the treatment for the treatment of psoriasis. One embodiment provides for pharmaceutical compositions comprising anti-human CD11a antibodies.

Apomab Antibodies

Antibodies to the DR5 receptor (anti-DR5) antibodies can also be produced in accordance with the present invention. Such anti-DR5 antibodies specifically include all antibody variants disclosed in PCT Publication No. WO 2006/083971, such as the anti-DR5 antibodies designated Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3, especially Apomab 8.3 and Apomab 7.3, preferably Apomab 7.3. The entire content of WO 2006/083971 is hereby expressly incorporated by reference. Apomab is a fully human monoclonal antibody which is a DR5-targeted pro-apoptotic receptor agonist (PARA) specifically designed to induce apoptosis. Apoptosis is a natural process by which damaged or unwanted cells, including those that are cancerous, die and are cleared from the body. Pro-apoptotic receptor DR5 is expressed in a broad range of malignancies.

Anti-BR3 Antibodies and Immunoadhesins

Antibodies to the BR3 (anti-BR3) antibodies and BR3-Fc immunoadhesins can also be produced in accordance with the present invention. Such anti-BR3 antibodies and immunoadhesins specifically include all variants disclosed in U.S. Application Publication No. 20050070689. The entire content of U.S. Application Publication No. 20050070689 is hereby expressly incorporated by reference.

3. General Methods for the Recombinant Production of Antibodies

The antibodies and other recombinant proteins herein can be produced by well known techniques of recombinant DNA technology. Thus, aside from the antibodies specifically identified above, the skilled practitioner could generate antibodies directed against an antigen of interest, e.g., using the techniques described below.

Antigen Selection and Preparation

The antibody herein is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those proteins described in section (3) below. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20, CD22, CD34, CD40; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA-4, ICAM-1, VCAM and αvγ/β3 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C, or any of the other antigens mentioned herein. Antigens to which the antibodies listed above bind are specifically included within the scope herein.

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of antigen or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal Antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the

selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, Protein A-Sepharose, hydroxyapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. Preferably the Protein A chromatography procedure described herein is used.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl Acad. Sci. USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

In a further embodiment, monoclonal antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional hybridoma techniques for isolation of monoclonal antibodies.

Humanized and Human Antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human FR for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immnol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of

producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and Duchosal et al., *Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 (1991); Vaughan et al., *Nature Biotech* 14:309 (1996)).

Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv) (see WO 93/16185).

Multispecific Antibodies

Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994). Alternatively, the antibodies can be

"linear antibodies" as described in Zapata et al., *Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

Immunoadhesins

The simplest and most straightforward immunoadhesin design combines the binding domain(s) of the adhesin (e.g. the extracellular domain (ECD) of a receptor) with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the immunoadhesins of the present invention, nucleic acid encoding the binding domain of the adhesin will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, C_H2 and C_H3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the C_H1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics of the immunoadhesin.

In a preferred embodiment, the adhesin sequence is fused to the N-terminus of the Fc domain of immunoglobulin G_1 (IgG_1). It is possible to fuse the entire heavy chain constant region to the adhesin sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (i.e. residue 216, taking the first residue of heavy chain constant region to be 114), or analogous sites of other immunoglobulins is used in the fusion. In a particularly preferred embodiment, the adhesin amino acid sequence is fused to (a) the hinge region and C_H2 and C_H3 or (b) the C_H1, hinge, C_H2 and C_H3 domains, of an IgG heavy chain.

For bispecific immunoadhesins, the immunoadhesins are assembled as multimers, and particularly as heterodimers or heterotetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG,

IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of four basic units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each of the four units may be the same or different.

Various exemplary assembled immunoadhesins within the scope herein are schematically diagrammed below:

AC_L-AC_L;

AC_H-(AC_H, AC_L-AC_H, AC_L-V_HC_H, or V_LC_L-AC_H);

AC_L-AC_H-(AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H)

AC_L-V_HC_H-(AC_H, or AC_L-V_HC_H, or V_LC_L-AC_H);

V_LC_L-AC_H-(AC_L-V_HC_H, or V_LC_L-AC_H); and

$(A\text{-}Y)_n$-$(V_LC_L\text{-}V_HC_H)_2$;

wherein each A represents identical or different adhesin amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_H is an immunoglobulin heavy chain constant domain;

n is an integer greater than 1;

Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed to be present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the adhesin sequences can be inserted between immunoglobulin heavy chain and light chain sequences, such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the adhesin sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the C_H2 domain, or between the C_H2 and C_H3 domains. Similar constructs have been reported by Hoogenboom, et al., *Mol. Immunol.* 28:1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an adhesin-immunoglobulin heavy chain fusion polypeptide, or directly fused to the adhesin. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the adhesin-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Pat. No. 4,816,567, issued 28 Mar. 1989.

Immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the adhesin portion in-frame to an immunoglobulin cDNA sequence. However, fusion to genomic immunoglobulin fragments can also be used (see, e.g. Aruffo et al., *Cell* 61:1303-1313 (1990); and Stamenkovic et al., Cell 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequences from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the "adhesin" and the immunoglobulin parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells.

Further details of the invention are provided in the following non-limiting Examples.

All patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties.

EXAMPLES

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples,

and throughout the specification, by ATCC® accession numbers is the American Type Culture Collection, Manassas, Va.

Example 1

Production of Polypeptides in Glutamine-Free Production Medium

Materials and Methods:

Cell Lines.

In these studies, CHO host cells expressing an Apomab antibody, anti-VEGF antibody, and the fusion protein BR3-Fc, respectively were used. The host cells were adapted in suspension and serum free cultures. Frozen stocks were prepared as master or working cell banks in the media described below.

Cell line maintenance was carried out using a 250-mL or 1-Liter Corning® vented shake flasks maintained in a Thermo Scientific Forma® reach-in a CO_2 humidified incubator maintained at 37° C. and 5% CO_2. Flasks were agitated at rate of 150 rpm on a New Brunswick Scientific Innova®-2100 platform shaker with a custom aluminum-substrate platform. Cell cultures were passed every 3 or 4 days with fresh media and seeded at 0.11% or 0.20% Packed Cell Volume (PCV). PCV was obtained using a glass10-mL KIMAX® USA PCV tube.

Culture Media and Conditions.

Media studies were initiated using 250-mL Corning vented shake flask inoculated in singlet, duplicate, or triplicate at 100 mL working volume at 0.20% PCV for all cases using cell culture from a source1-Liter Corning® vented shake flask with a 500-mL working volume. PCV was obtained using a glass10-mL KIMAX® USA PCV tube.

Prior to initiation of the study cell culture was centrifuged at 1000 rpm for 5-minutes in a Sorvall® RT 6000B centrifuge to complete a 100% media exchange of inoculum media containing glutamine with the respective test media. Different concentrations of Glutamine, Glutamate, Asparagine and Aspartate were evaluated in the different test media. The following concentrations were tested: Glutamine 0-10 mM, Glutamate 1-10 mM, Asparagine 0-15 mM, Aspartate 1-10 mM. Media conditions were evaluated in full factorial DOE studies.

The effect of Glutamine-free medium on was also tested in commercially available DMEM/F12 medium. The medium was used at 5× concentration (7.05 g/L) with extra Asparagine (10 mM total), Aspartate (10 mM total), Glutamine (10 mM total for the Glutamine-containing medium), Glutamate (1 mM total), and glucose (8 g/L total). Glutamine-free and Glutamine-containing medium were compared using Apomab and anti-VEGF antibody expressing cells.

Shake flasks were maintained in a Thermo Scientific Forma® reach-in a CO_2 humidified incubator maintained at 37® C. and 5% CO_2. Flasks were agitated at rate of 150 rpm on a New Brunswick Scientific Innova®-2100 platform shaker with a custom aluminum-substrate platform.

The medium used contained the following components:

Organic salts and Trace Elements
Ammonium Paramolybdate, Tetrahydrate
Ammonium Vanadium Oxide
Calcium Chloride, Anhydrous
Cupric Sulfate, Pentahydrate
Ferrous Sulfate, Heptahydrate

-continued

Potassium Chloride
Magnesium Chloride, Anhydrous
Manganese Sulfate, Monohydrate
Nickel Chloride, Hexahydrate
Selenious Acid
Sodium Metasilicate, Nonahydrate
Sodium Phosphate, Monobasic, Monohydrate
Stannous Chloride, Dihydrate
Zinc Sulfate, Heptahydrate
Lipids
Linoleic Acid
Lipoic Acid (aka Thioctic Acid)
Putrescine, Dihydrochloride
Amino Acids
L-Alanine
L-Arginine, Monohydrochloride
L-Asparagine
L-Aspartic Acid
L-Cysteine, Monohydrochloride, Monohydrate
L-Glutamic Acid
L-Glutamine
L-Histidine, Monohydrochloride, Monohydrate
L-Isoleucine
L-Leucine
L-Lysine, Monohydrochloride
L-Methionine
L-Phenylalanine
L-Proline
L-Serine
L-Threonine
L-Tryptophan
L-Tyrosine, Disodium Salt, Dihydrate
L-Valine
Vitamins
Biotin
D-Calcium Pantothenate
Choline Chloride
Folic Acid
I-Inositol
Niacinamide
Pyridoxine, Monohydrochloride
Riboflavin
Thiamine, Monohydrochloride
Vitamin B-12
Carbon Source, Growth Factors, and Miscelaneous
Fluronic F-68
D-Glucose
Sodium Bicarbonate
Sodium Pyruvate
Sodium Chloride
Sodium Hydroxide
Insulin
Galactose

The commercially-available DMEM/F-12 culture medium was also tested, having the following components;

	(mg/L)
VITAMINS	
Biotin	0.00365
D-calcium pantothenate	2.24
Choline chloride	8.98
Cyanocobalamin	0.68
Folic acid	2.65
i-inositol	12.6
Niacinamide	2.0185
Pyridoxal HCl	2
Pyridoxine HCl	0.031
Riboflavin	0.219
Thiamine HCl	2.17

-continued

	(mg/L)
AMINO ACIDS	
L-alanine	4.455
L-arginine HCl	147.5
L-asparagine monohydrate	7.5
L-aspartic acid	6.65
L-cysteine HCl monohydrate	17.56
L-cystine 2HCl	31.29
L-glutamic acid	7.35
L-glutamine	365
Glycine	18.75
L-histidine HCl monohydrate	31.48
L-isoleucine	54.47
L-leucine	59.05
L-lysine HCl	91.25
L-methionine	17.24
L-phenylalanine	35.48
L-proline	17.25
L-serine	26.25
L-threonine	53.45
L-tryptophan	9.02
L-tyrosine 2Na dihydrate	55.79
L-valine	52.85
OTHER	
Dextrose anhydrous	3151
HEPES	3575
Hypoxanthine sodium salt	2.39
Linoleic acid	0.042
DL-α-Lipoic acid	0.105
Phenol red sodium salt	8.602
Putrescine 2HCl	0.081
Sodium pyruvate	55
Thymidine	0.365
ADD: Sodium bicarbonate	1200
INORGANIC SALTS	
Calcium chloride anhydrous	116.61
Cupric sulfate pentahydrate	0.00125
Ferric nitrate nonahydrate	0.05
Ferrous sulfate heptahydrate	0.417
Magnesium chloride anhydrous	28.61
Magnesium sulfate anhydrous	48.84
Potassium chloride	311.8
Sodium chloride	6999.5
Sodium phosphate dibasic anhydrous	71.02
Sodium phosphate monobasic monohydrate	62.5
Zinc sulfate heptahydrate	0.4315

The medium for inoculum culture (as opposed for the production phase) was usually supplemented with 5 mM glutamine, 8 g/L glucose, and 75-2000 nM Methotroxate.

For studies pH adjustment was performed as needed to maintain pH value at 7.00±0.10 using 1M Sodium Carbonate. Adjustment in pH value was made in by adding 1 mL/L of 1M Sodium Carbonate to raise pH units up 0.10.

Cell culture was analyzed up to 14-days by taking a 3.5-mL sample and analyzed for viable cell count, viability, and cell size using a Beckman Coulter ViCell™-1.0 cell counter. Nutrient analysis was performed using the Nova 400 Biomedical Bioprofile®. Osmolality was measured using an Advanced® Instrument multi-sample Osmometer (Model 3900). Recombinant product titer concentration was obtained using the Agilent 1100 Series HPLC.

Recombinant Proteins.

The recombinant proteins produced were Apomab (TRAIL), anti-VEGF, and the immunoadhesin BR3-Fc.

Data Analysis.

Statistical analyses of the data were carried out using a full factorial design of experiment, which is an experiment whose design consists of two or more factors, each with discrete possible values or "levels", and whose experimental units take on all possible combinations of these levels across all such factors. A full factorial design may also be called a fully-crossed design. Such an experiment allows studying the effect of each factor on the response variable, as well as the effects of interactions between factors on the response variable.

Results

As shown in FIGS. **1-5**, use of a glutamine-free production medium increased the final recombinant protein titer of Apomab antibody, BR3-Fc immunoadhesin and anti-VEGF antibody. In each case, cube plot analysis of titer results using Full Factorial DOE evaluating the effect of different concentrations of Glutamine, Glutamate, Asparagine and Aspartate predict that the highest titer is achieved in Glutamine-Free media supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid. (FIGS. **1-3**)

The effect of Asparagine under Glutamine-free, low Glutamate and high Aspartate conditions on Apomab antibody titer is shown in FIG. **4**. In Glutamine-free medium, Apomab antibody titer was significantly increased in the presence of 2.5-15 mM Asparagine compared to Glutamine-free cultures without Asparagine. Under these conditions, the presence or absence of Glutamate had no effect on titer.

Apomab antibody titer production across various Asparagine and Aspartate concentrations in Glutamine-free and low Glutamate conditions is illustrated in FIG. **5**. A positive titration effect was observed when increasing Aspartate from 0 to 10 mM under these conditions.

The effect of glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid on titer is demonstrated in FIGS. **6** A-C, wherein the final titer for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin (A-C, respectively) was significantly higher in Glutamine-free medium compared to Glutamine-containing medium.

Similar results were obtained using the commercial DMEM/F-12 culture medium. As shown in FIGS. **7** A and B, the final titer for Apomab antibody and anti-VEGF antibody (A and B, respectively) was significantly higher in Glutamine-free DMEM/F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing DMEM F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid.

As shown in FIGS. **8** and **9**, use of a glutamine-free production medium also increased specific production measured as Qp (mg/mL-cell/day). FIGS. **8** A-C illustrate that cell specific productivity (Qp) for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin (A-C, respectively) was significantly higher in Glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing medium. FIGS. **9** A and B illustrate that cell specific productivity for Apomab antibody and anti-VEGF antibody (A and B, respectively) was significantly higher in Glutamine-free DMEM/F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing DMEM/F12 medium.

As shown in FIGS. **10** and **11**, use of a glutamine-free production medium was shown to improve cell viability and extend culture longevity significantly. FIGS. **10** A-C. illustrate that cell viability for Apomab antibody, anti-VEGF antibody and BR3-Fc immunoadhesin (A-C, respectively) was higher in Glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic

Acid compared to Glutamine-containing medium. FIGS. **11** A and B indicate that, in DMEM/F12 medium, cell viability was not consistently improved in Glutamine-free medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid. Of note, viability was higher for Apomab antibody (FIG. **11** A), but lower for anti-VEGF antibody (FIG. **11** B) compared to Glutamine containing medium.

As shown in FIGS. **12** and **13**, use of a glutamine-free production medium reduced NH_4^+ accumulation significantly compared to glutamine-containing medium. FIGS. **12** A-D illustrate that ammonia levels were usually lower in Glutamine-free cultures supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing cultures. FIGS. **13** A and B illustrate that ammonia levels were significantly reduced in Glutamine-free DMEM/F12 medium supplemented with 10 mM Asparagine, 10 mM Aspartic Acid and 1 mM Glutamic Acid compared to Glutamine-containing DMEM/F12 medium.

The invention illustratively described herein can suitably be practiced in the absence of any element or elements, limitation or limitations that is not specifically disclosed herein. Thus, for example, the terms “comprising,” “including,” “containing,” etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalent of the invention shown or portion thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modifications and variations of the inventions embodied herein disclosed can be readily made by those skilled in the art, and that such modifications and variations are considered to be within the scope of the inventions disclosed herein.

From the description of the invention herein, it is manifest that various equivalents can be used to implement the concepts of the present invention without departing from its scope. Moreover, while the invention has been described with specific reference to certain embodiments, a person of ordinary skill in the art would recognize that changes can be made in form and detail without departing from the spirit and the scope of the invention. The described embodiments are considered in all respects as illustrative and not restrictive. It should also be understood that the invention is not limited to the particular embodiments described herein, but is capable of many equivalents, rearrangements, modifications, and substitutions without departing from the scope of the invention. Thus, additional embodiments are within the scope of the invention and within the following claims.

All U.S. patents and applications; foreign patents and applications; scientific articles; books; and publications mentioned herein are hereby incorporated by reference in their entirety as if each individual patent or publication was specifically and individually indicated to be incorporated by reference, including any drawings, figures and tables, as though set forth in full.

What is claimed is:

1. A process for producing a polypeptide in a host cell expressing said polypeptide, comprising culturing the host cell in a production phase of the culture in a glutamine-free production culture medium containing asparagine and aspartic acid, wherein the asparagine is added at a concentration in the range of 7.5 mM to 15 mM and wherein the aspartic acid is added at a concentration in the range of 1 mM to 10 mM.

2. The process of claim **1** further comprising the step of isolating said polypeptide.

3. The process of claim **2** further comprising determining one or more of cell viability, culture longevity, specific productivity and final recombinant protein titer following isolation.

4. The process of claim **3** wherein at least one of the cell viability, culture longevity, specific productivity and final recombinant protein titer is increased relative to the cell viability, culture longevity, specific productivity and final recombinant protein titer in a glutamine-containing production medium of the same composition.

5. The process of claim **1** wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

6. The process of claim **1** wherein the asparagine is added at a concentration of 10 mM.

7. The process of claim **1** wherein the production medium is serum-free.

8. The process of claim **1** wherein the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

9. The process of claim **8** wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

10. The process of claim **1** wherein the production phase is a batch or fed batch culture phase.

11. The process of claim **10**, wherein the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

12. The process of claim **11**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

13. The process of **11**, wherein the asparagine is added at a concentration of 10 mM.

14. The process of claim **11**, wherein the aspartic acid is added at a concentration of 10 mM.

15. The process of claim **11**, wherein the production medium is serum-free.

16. The process of claim **10**, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

17. The process of claim **16**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

18. The process of claim **16**, wherein the asparagine is added at a concentration of 10 mM.

19. The process of claim **16**, wherein the aspartic acid is added at a concentration of 10 mM.

20. The process of claim **16**, wherein the production medium is serum-free.

21. The process of claim **1** wherein said host cell is an eukaryotic host cell.

22. The process of claim **21** wherein said eukaryotic host cell is a mammalian host cell.

23. The process of claim **22**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

24. The process of claim **22**, wherein the asparagine is added at a concentration of 10 mM.

25. The process of claim **22**, wherein the aspartic acid is added at a concentration of 10 mM.

26. The process of claim **22**, wherein the production medium is serum-free.

27. The process of claim **22**, wherein the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

28. The process of claim **27**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

29. The process of claim **27**, wherein the asparagine is added at a concentration of 10 mM.

30. The process of claim **27**, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

31. The process of claim **22**, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

32. The process of claim **31**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

33. The process of claim **31**, wherein the asparagine is added at a concentration of 10 mM.

34. The process of claim **31**, wherein the aspartic acid is added at a concentration of 10 mM.

35. The process of claim **31**, wherein the production medium is serum-free.

36. The process of claim **22**, wherein the production phase is a batch or fed batch culture phase.

37. The process of claim **36**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

38. The process of claim **36**, wherein the asparagine is added at a concentration of 10 mM.

39. The process of claim **36**, wherein the aspartic acid is added at a concentration of 10 mM.

40. The process of claim **36**, wherein the production medium is serum-free.

41. The process of claim **22** wherein said mammalian host cell is a Chinese Hamster Ovary (CHO) cell.

42. The process of claim **41** wherein the mammalian host cell is a dhfr− CHO cell.

43. The process of claim **41**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

44. The process of claim **41**, wherein the asparagine is added at a concentration of 10 mM.

45. The process of claim **41**, wherein the aspartic acid is added at a concentration of 10 mM.

46. The process of claim **41**, wherein the production medium is serum-free.

47. The process of claim **41**, wherein the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

48. The process of claim **47**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

49. The process of claim **47**, wherein the asparagine is added at a concentration of 10 mM.

50. The process of claim **47**, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

51. The process of claim **41**, wherein the production culture medium additionally comprises one or more ingredients selected from the group consisting of:

1) hormones and other growth factors;
2) salts and buffers; and
3) nucleosides.

52. The process of claim **51**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

53. The process of claim **51**, wherein the asparagine is added at a concentration of 10 mM.

54. The process of claim **51**, wherein the aspartic acid is added at a concentration of 10 mM.

55. The process of claim **51**, wherein the production medium is serum-free.

56. The process of claim **41**, wherein the production phase is a batch or fed batch culture phase.

57. The process of claim **56**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

58. The process of claim **56**, wherein the asparagine is added at a concentration of 10 mM.

59. The process of claim **56**, wherein the aspartic acid is added at a concentration of 10 mM.

60. The process of claim **56**, wherein the production medium is serum-free.

61. The process of claim **56**, wherein the production culture medium comprises one or more ingredients selected from the group consisting of

1) an energy source;
2) essential amino acids;
3) vitamins;
4) free fatty acids; and
5) trace elements.

62. The process of claim **61**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

63. The process of claim **61**, wherein the asparagine is added at a concentration of 10 mM.

64. The process of claim **61**, wherein the aspartic acid is added at a concentration of 10 mM.

65. The process of claim **1** wherein the polypeptide is a mammalian glycoprotein.

66. The process of claim **1** wherein the polypeptide is selected from the group consisting of antibodies, antibody fragments, and immunoadhesins.

67. The process of claim **66** wherein said antibody fragment is selected from the group consisting of Fab, Fab', F(ab')2, scFv, (scFv)2, dAb, complementarity determining region (CDR) fragments, linear antibodies, single-chain antibody molecules, minibodies, diabodies, and multispecific antibodies formed from antibody fragments.

68. The process of claim **66** wherein the antibody or antibody fragment is chimeric, humanized or human.

69. The process of claim **66** wherein said antibody or antibody fragment is a therapeutic antibody or a biologically functional fragment thereof.

70. The process of claim **69** wherein said therapeutic antibody is selected from the group consisting of anti-HER2 antibodies; anti-CD20 antibodies; anti-IL-8 antibodies; anti-VEGF antibodies; anti-CD40 antibodies; anti-CD 11 a antibodies; anti-CD 18 antibodies; anti-IgE antibodies; anti-Apo-2 receptor antibodies; anti-Tissue Factor (TF) antibodies; anti-human $\alpha 4\beta 7$ integrin antibodies; anti-EGFR antibodies; anti-CD3 antibodies; anti-CD25 antibodies; anti-CD4 antibodies; anti-CD52 antibodies; anti-Fc receptor antibodies; anti-carcinoembryonic antigen (CEA) antibodies; antibodies directed against breast epithelial cells; antibodies that bind to colon carcinoma cells; anti-CD38 antibodies; anti-CD33 antibodies; anti-CD22 antibodies; anti-EpCAM antibodies; anti-GpIIb/IIIa antibodies; anti-RSV antibodies; anti-CMV antibodies; anti-HIV antibodies; anti-hepatitis antibodies; anti-CA 125 antibodies; anti-$\alpha v\beta 3$ antibodies; anti-human renal cell carcinoma antibodies; anti-human 17-1A antibodies; anti-human colorectal tumor antibodies; anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma; and anti-human leukocyte antigen (HLA) antibodies, and anti-HLA DR antibodies.

71. The process of claim **69** wherein said therapeutic antibody is an antibody binding to a HER receptor, VEGF, IgE, CD20, CD11a, CD40, BR3 or DR5.

72. The process of claim **71**, wherein the therapeutic antibody is selected from the group consisting of bevacizumab, rituximab, and trastuzumab.

73. The process of claim **72**, wherein the asparagine is added at a concentration in the range of 7.5 mM to 10 mM.

74. The process of claim **72**, wherein the asparagine is added at a concentration of 10 mM.

75. The process of claim **72**, wherein the aspartic acid is added at a concentration of 10 mM.

76. The process of claim **72**, wherein the production medium is serum-free.

77. The process of claim **1** wherein said polypeptide is a therapeutic polypeptide.

78. The process of claim **77** wherein said therapeutic polypeptide is selected from the group consisting of a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-$\beta 1$, TGF-$\beta 2$, TGF-$\beta 3$, TGF-$\beta 4$, or TGF-$\beta 5$; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of said polypeptides.

* * * * *

EXHIBIT II

US008357301B2

(12) **United States Patent**
Belousov et al.

(10) **Patent No.: US 8,357,301 B2**
(45) **Date of Patent: Jan. 22, 2013**

(54) **CHROMATOGRAPHY EQUIPMENT CHARACTERIZATION**

(75) Inventors: **Anton Belousov**, Penzberg (DE); **Thomas Dams**, Pullach (DE); **Benjamin Gerwat**, Weilheim i. OB (DE)

(73) Assignee: **Hoffmann-La Roche, Inc.**, Nutley, NJ (US)

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PCT Pub. Date: **Dec. 29, 2010**

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B01D 15/08 (2006.01)

(52) **U.S. Cl.** **210/656**; 210/198.2; 530/413; 702/30; 702/84

(58) **Field of Classification Search** 210/635, 210/656, 657, 659, 143, 198.2; 702/30, 81, 702/84, 104; 530/413
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,456,955 B1 * 9/2002 Andrews et al. 702/104
(Continued)

FOREIGN PATENT DOCUMENTS

WO 02/47009 6/2002

OTHER PUBLICATIONS

Sarker et al., "Study of the packaging behavior of axial compression columns for preparative chromatography" Journal of Chromatography A 702:27-44 (1995).
Larson et al., "Use of Process Data to Assess Chromatographic Performance in Production-Scale Protein Purification Columns" Biotechnol 19(2):485-492 (2003).

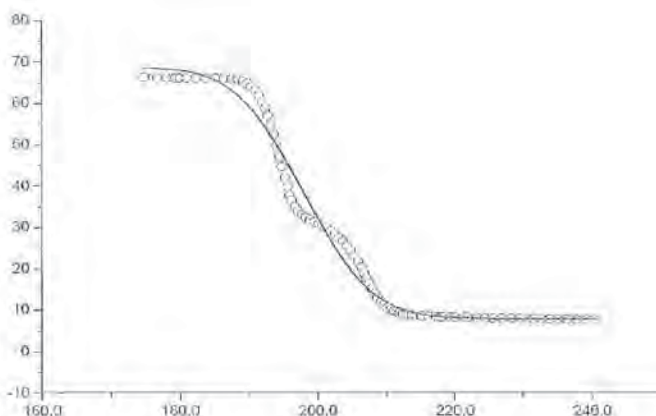
(Continued)

Primary Examiner — Ernest G. Therkorn
(74) *Attorney, Agent, or Firm* — Janet M. Martineau

(57) **ABSTRACT**

Herein is reported a method for determining whether a re-useable chromatography column packing, which is used at least for the second time in a purification step of a purification of a polypeptide, has reduced separation efficacy in said purification step of said purification of said polypeptide, comprising the following steps: a) identifying and determining the experimental data of an inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing, b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use, c) determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b), d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference, e) determining reduced separation efficacy of said re-useable chromatography column packing when the absolute value of the difference calculated in step d) is more than 0.1.

18 Claims, 8 Drawing Sheets



U.S. PATENT DOCUMENTS

7,132,053	B2 *	11/2006	Hauck et al.	210/656
7,267,765	B2 *	9/2007	Hauck et al.	210/198.2
7,890,285	B2 *	2/2011	Manfredi	702/120
8,163,177	B2 *	4/2012	Van Pelt	210/198.2
2010/0084344	A1 *	4/2010	Lihme et al.	210/656
2011/0147312	A1 *	6/2011	Cunnien et al.	210/656

OTHER PUBLICATIONS

Moscariello et al., "Charaterizing the performance of industial-scale columns" Journal of Chromatography A 908:131-141 (2001).
Vink et al., "Resolution and Column Efficiency in Chromatography" Journal of Chromatography 69:237-242 (1972).

* cited by examiner

Fig. 1

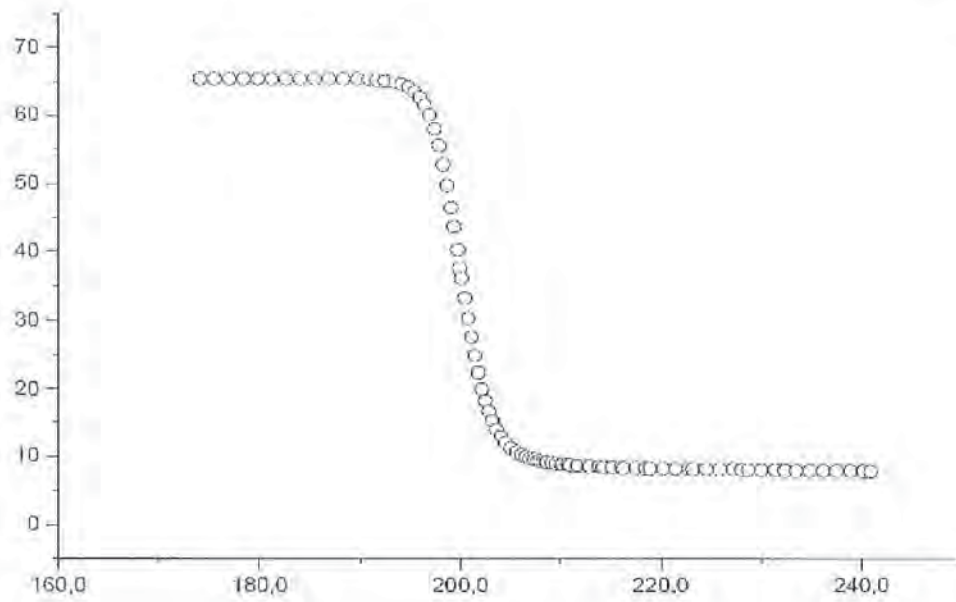


Fig. 2

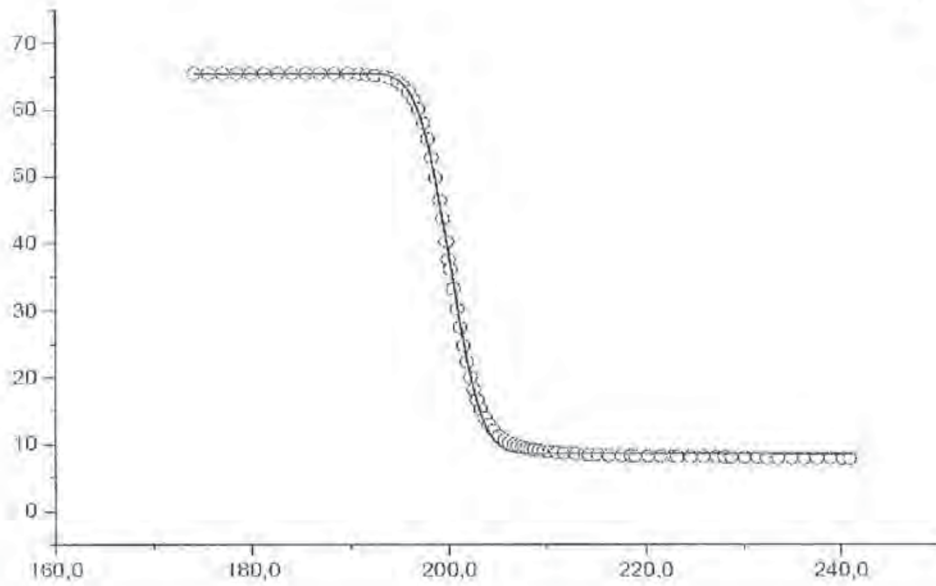


Fig. 3

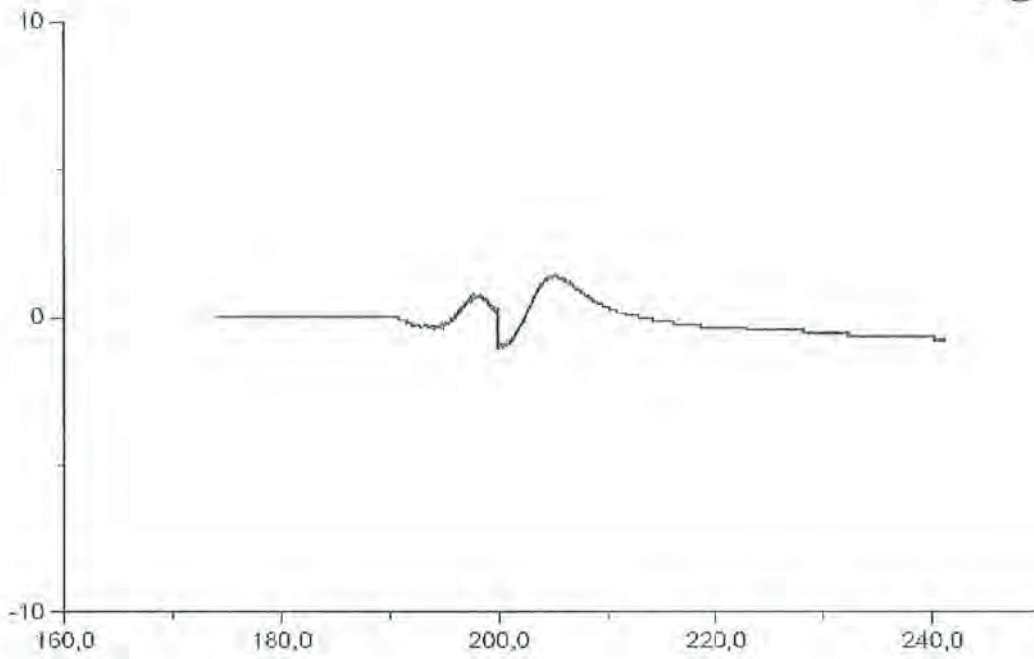


Fig. 4

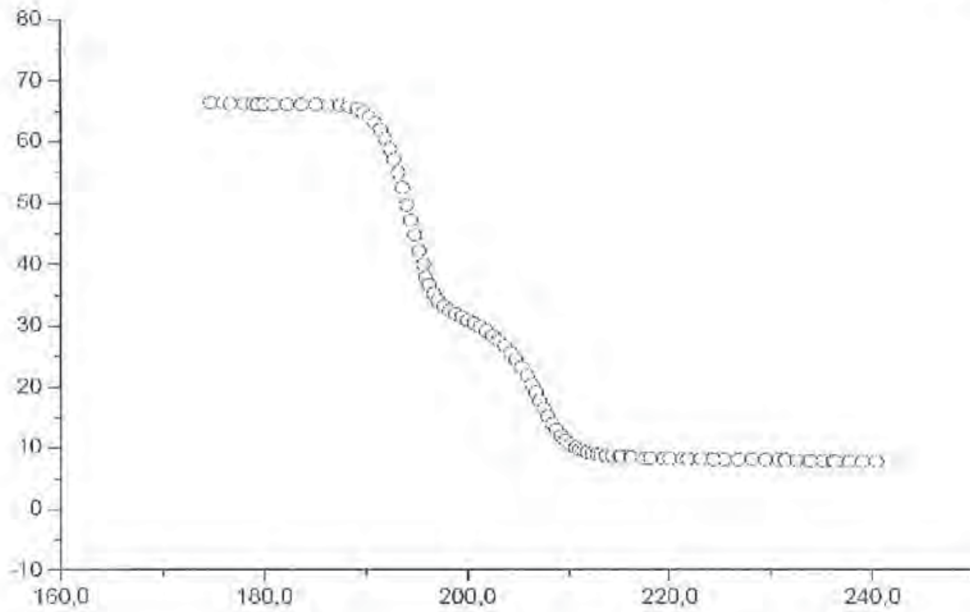


Fig. 5

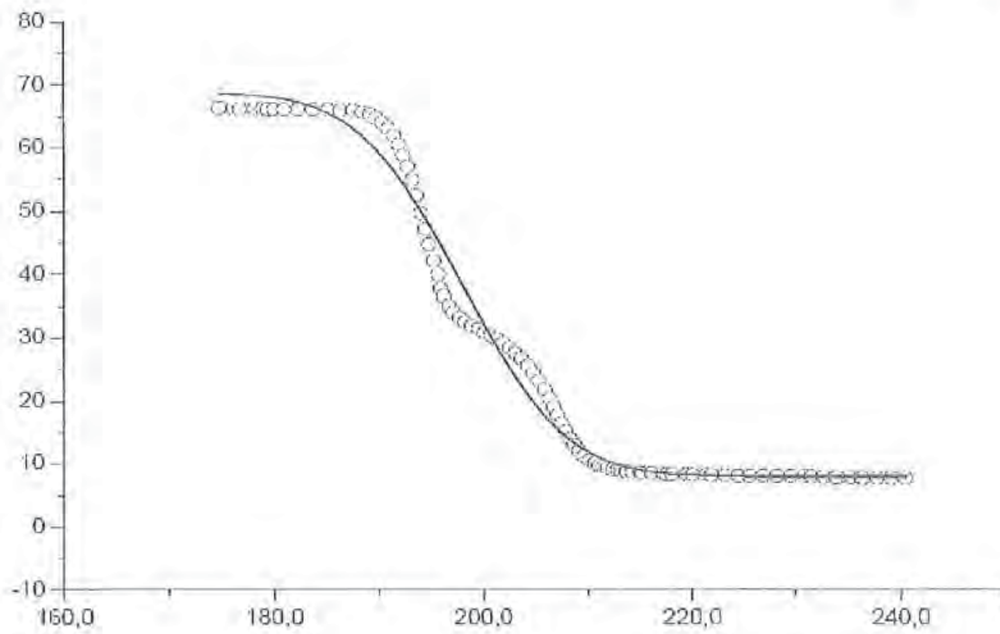


Fig. 6

Fig. 7

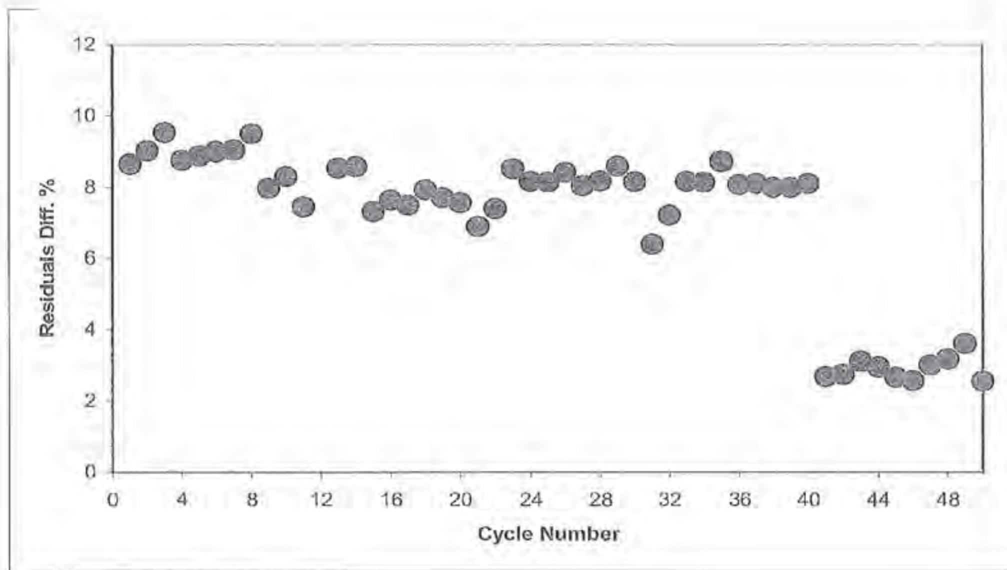
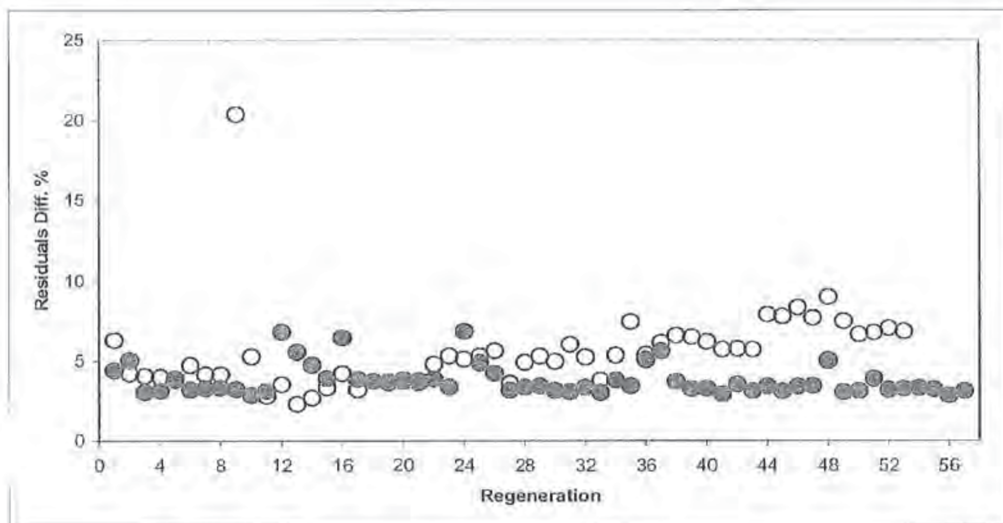


Fig. 8



CHROMATOGRAPHY EQUIPMENT CHARACTERIZATION

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a National Phase filing under 35 U.S.C. §371 of International Application No. PCT/EP2010/003813 having an international filing date of Jun. 22, 2010, the entire contents of which are incorporated herein by reference, and which claim benefit under 35 U.S.C. §119 to European Patent Application No. 09008247.0 filed Jun. 24, 2009.

The herein reported method is in the field of chromatography, especially in the field of preparative column chromatography. It is herein reported a method for the direct determination of the quality of the packing of a chromatography column based on in process data. With this method a saving in process time and resources can be achieved as an additional data acquisition solely for the purpose of column integrity determination can be eliminated.

BACKGROUND OF THE INVENTION

Today almost all polypeptides used in medicaments are prepared recombinantly. Due to strict regulatory guidelines and requirements by-products have to be removed from the therapeutic polypeptide preparation as much as possible. Therefore, at least one chromatography step is employed in down stream processing of the bulk raw polypeptide after recombinant production. As the dimension of the chromatography equipment with respect to the yield of the fermentation process, especially the separation capacity of chromatography columns, is limited, a multitude of batches have to be processed in order to be able to provide the required amount of purified therapeutic polypeptide.

To ensure that each batch of the purified therapeutic polypeptide has the same pharmaceutical effect, a list of analytical parameters has to be fulfilled for each batch. This can only be achieved if the steps of the purification process operate consistently and efficiently. But, if one step of the purification process does not work properly the obtained product will most probably not pass the analytical tests and, in the worst case, this batch cannot be used. Therefore, it is necessary to provide methods for determining the performance and efficacy of purification steps.

Teeters, M. A. and Quinones-Garcia, I. (J. Chrom. A 1069 (2005) 53-64) report the evaluating and monitoring the packing behavior of process-scale chromatography columns by using the responses to conductivity-based pulse and step inputs derived from tracer experiments and in-process transitions, especially from measured residence time distributions. Norling et al. (Norling, L., et al. J. Chrom. A 1069 (2005) 79-89) report the impact of multiple re-use of anion-exchange chromatography media on virus removal. The use of process data to assess chromatographic performance in production-scale protein purification columns is reported by Larson et al. (Larson, T. M., et al., Biotechnol. Prog. 19 (2003) 485-492). Moscariello, J., et al. J. Chrom. A 908 (2001) 131-141 report the characterization of the performance of industrial-scale columns. The resolution and column efficiency in chromatography is reported by Vink, H., J. Chrom. 69 (1972) 237-242. Sarker, M. and Guiochon, G., J. Chrom. A 702 (1995) 27-44 report a study of the packing behavior of axial compression columns for preparative chromatography.

SUMMARY OF THE INVENTION

With the method as reported herein a determination of the decrease in the separation efficacy and/or packing quality of a re-useable chromatography column packing can be determined without the need to use and inject a further tracer compound prior to the separation of the crude polypeptide solution for the determination of column material integrity or the need for historical data of this purification step.

The first aspect as reported herein is a method for determining whether a re-useable chromatography column packing, which is used at least for the second time in a purification step of a purification of a polypeptide, has reduced separation efficacy, e.g. compared to the separation efficacy when it was used for the first time in the same purification step of the same purification of the same polypeptide, comprising the following steps:

a) identifying and determining the experimental data of an inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing,
b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use,
c) determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b),
d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference,
e) determining reduced separation efficacy of said re-useable chromatography column packing when the absolute value of the difference calculated in step d) is more than 0.1,

wherein the function of formula I is

$$yI = \frac{1}{2}P1 \cdot \left(1 + \mathrm{erf}\left(\frac{x-m}{s \cdot \sqrt{2}}\right)\right) + A0,$$

with the amplitude P1, the starting value A0, the mean value m, the standard deviation s, and with

$$\mathrm{erf}(x) = \frac{2}{\sqrt{\pi}} \sum_{0}^{\infty} \frac{(-1)^n x^{2n+1}}{(2n+1)n!}.$$

Another aspect as reported herein is a method for the chromatographic purification of a polypeptide, in which at least one chromatography step with a re-useable chromatography column packing is comprised, comprising the following steps:

a) identifying and determining the experimental data of an inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing,
b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use,
c) determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b),
d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference,

wherein the function of formula I is

$$yI = \frac{1}{2}P1 \cdot \left(1 + \text{erf}\left(\frac{x-m}{s \cdot \sqrt{2}}\right)\right) + A0,$$

with the amplitude P1, the starting value A0, the mean value m, the standard deviation s, and with

$$\text{erf}(x) = \frac{2}{\sqrt{\pi}} \sum_{0}^{\infty} \frac{(-1)^n x^{2n+1}}{(2n+1)n!},$$

and

further using the re-useable chromatography column packing when the absolute value of the difference calculated in step d) is 0.05 or less, or

performing additional characterization of the purified polypeptide when the absolute value of the difference calculated in step d) is more than 0.05 but less than 0.2, or

changing the re-useable chromatography column packing when the absolute value of the difference calculated in step d) is 0.2 or more.

In one embodiment said inert change of at least one physicochemical parameter of said mobile phase passing through said re-useable chromatography column packing is a significant signal change effected by the change of the concentration of a substance that does not interact with the re-useable column packing contained in said mobile phase. In another embodiment said determining the experimental data is a determining over time the experimental data of a physicochemical parameter of an inert change. In a further embodiment said inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing is a change of the mobile phase from 100% of a solution containing a denaturing agent to 100% of a solution not containing said denaturing agent or from 100% of a solution not containing said denaturing agent to 100% of a solution containing a denaturing agent. In another embodiment the denaturing agent is selected from sodium hydroxide, guanidinium chloride, urea, or organic solvent. In one embodiment said step c) is determining the differences between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b) for each experimental data point. In one embodiment said signal change is a change in conductivity or in adsorption at 280 mn. In a further embodiment said inert change is a sigmoid change. In still another embodiment said at least one physicochemical parameter is determined in the conditioning or regeneration step.

DETAILED DESCRIPTION OF THE INVENTION

The first aspect as reported herein is a method for determining whether a re-useable chromatography column packing, which is used at least for the second time in a purification step of a purification of a polypeptide, has reduced separation efficacy compared to the separation efficacy when it was used for the first time in the same purification step of the same purification of the same polypeptide, comprising the following steps:

a) identifying an inert change and determining over time the experimental data of a physicochemical parameter of an inert change from 100% of a solution containing a denaturing agent to 100% of a solution not containing said denaturing agent, or vice versa, of a mobile phase passing through said re-useable chromatography column packing after the at least first use of the chromatography column packing,

b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use obtained in a),

c) determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b),

d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference,

e) determining reduced separation efficacy of said re-useable chromatography column packing when the absolute value of the difference calculated in step d) is more than 0.05,

wherein the function of formula I is

$$yI = \frac{1}{2}P1 \cdot \left(1 + \text{erf}\left(\frac{x-m}{s \cdot \sqrt{2}}\right)\right) + A0,$$

with the amplitude P1, the starting value A0, the mean value m, the standard deviation s, and with

$$\text{erf}(x) = \frac{2}{\sqrt{\pi}} \sum_{0}^{\infty} \frac{(-1)^n x^{2n+1}}{(2n+1)n!}.$$

The term "re-useable chromatography column packing" denotes a chromatography material that is packed into a chromatography column whereby the chromatography material is obtained after a purification in a not modified form, i.e. with the same characteristics as prior to the purification. A purification step denotes in general a cycle comprising the conditioning of the chromatography column packing, the application of the crude polypeptide solution, optionally the washing of the chromatography material, the recovery of the purified polypeptide from the chromatography column packing and the regeneration of the chromatography column packing. In one embodiment of the aspects as reported herein the inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing is a change of a physicochemical parameter over time and/or in the conditioning of the chromatography column packing and/or in the regeneration of the chromatography column packing.

The definition of a re-useable chromatography column packing as outlined above requires that all individual steps of a purification are perfectly reversible. But this is not the case. During the purification step e.g. the completely homogeneous nature of the packed chromatography material may become disturbed and the flow through the separation matrix can be compromised. At one point in time the separation efficacy and/or recovery and/or packing quality of the re-useable chromatography material is still sufficient to allow for a purification of the polypeptide from by-products but not in a purity

fulfilling the requirements of the specification of said polypeptide. As a result this batch of the polypeptide maybe cannot be used as a therapeutic and has to be further treated or discarded.

With the method as reported herein a determination of the decrease in the separation efficacy and/or packing quality of and/or in the recovery from a re-useable chromatography column packing is possible without i) the need to use and inject a further tracer compound prior to the separation of the crude polypeptide solution for the determination of column material integrity or ii) the need for historical data of this purification step. Thus, the method according to the current invention allows for the determination of the quality of a re-useable chromatography column packing based on data that is generally obtained during the chromatography purification step of the polypeptide making additional steps such as tracer substance injection unnecessary.

The method as reported herein is based on the finding that an inert change of at least one physicochemical parameter of a mobile phase passing through a re-useable chromatography column packing during the purification of a polypeptide can be used for determining the chromatography material separation efficacy and/or packing quality. Such an "inert change" is the change of at least one, preferably one, physicochemical parameter over time, such as the concentration of a substance contained in the mobile phase, or of the mobile phase itself during the purification step. The substance does not interact with the functionality of the chromatography material effecting the purification of the polypeptide. Exemplary inert changes of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing are i) a change from denaturing conditions to non-denaturing conditions, or ii) a change from strongly alkaline conditions to buffered conditions, or iii) a change from organic solvent to water. In one embodiment the change is from 100% of a 0.5 to 1 M sodium hydroxide solution or a 5 M guanidinium chloride solution or an 8 M urea solution or organic solvent to 100% buffer or 100% water, optionally comprising an ionizing agent such as trifluoro acetic acid in up to 1% (v/v). Or vice versa the changes is i) from non-denaturing conditions to denaturing conditions, or ii) from buffered conditions to strongly alkaline conditions, or iii) from water to organic solvent. In another embodiment the change is from 100% buffer or 100% water, optionally comprising an ionizing agent such as trifluoro acetic acid in up to 1% (v/v), to 100% of a 0.5 to 1 M sodium hydroxide solution, or to 100% of a 5 M guanidinium chloride solution, or to 100% of an 8 M urea solution or organic solvent. A chromatogram for an inert change showing no reduction in separation efficacy/packing quality is shown in FIG. **1** and a chromatogram for an inert change showing a reduction in separation efficacy/packing quality is shown in FIG. **4**.

In one embodiment the inert change of the at least one physicochemical parameter over time is determined by the experimental data recorded during the purification, such as the absorption at 280 mn, or the conductivity of the mobile phase leaving the chromatography column, or the organic solvent concentration leaving the chromatography column.

The term "mobile phase" denotes a liquid that is used in column chromatography and that surrounds the chromatography material of the chromatography column packing, which in turn is the stationary phase.

It has been found that from a comparison of the experimental data recorded during the inert change of the at least second use of said re-useable chromatography column packing with the recorded data during the inert change of the at least second use of said re-useable chromatography column packing fitted to the function of formula I a determination of the separation efficacy/packing quality of the re-useable chromatography column packing can be obtained. In one embodiment the experimental data is recorded during the at least second purification of said polypeptide with a column packing made with chromatography material that has been used one or more times in the same purification steps of the same purification of the same polypeptide prior to this use. The function of formula I is as follows:

$$yI = \frac{1}{2}P1 \cdot \left(1 + \operatorname{erf}\left(\frac{x-m}{s \cdot \sqrt{2}}\right)\right) + A0,$$

with

the amplitude P1,

the starting value A0,

the mean value m,

the standard deviation s,

and with

$$\operatorname{erf}(x) = \frac{2}{\sqrt{\pi}} \sum_{0}^{\infty} \frac{(-1)^n x^{2n+1}}{(2n+1)n!}.$$

The methods as reported herein employ the integrated form of the Gaussian distribution yielding quality attributes for the packing of the column. With the function of formula I the deviation from a purely diffusion controlled distribution mechanism of a compound(s) contained in an approximately rectangular pulse of said compound(s) at the column inlet can be determined. This is achieved by comparing the function of formula I fitted to the experimental data recorded during the identified inert change in the purification of the polypeptide with the recorded experimental data itself, whereby the difference during the inert change of the fitted function and the not-fitted experimental data should not exceed a predetermined threshold in order to provide a purified polypeptide of desired characteristics.

The comparison is done by calculating the difference between the experimental data with which the function of formula I has been fitted and the fitted function of formula I. In order to make the results of individual purifications comparable to each other the results or the difference are normalized, e.g. by dividing the value with the maximum value of the experimental data of said physicochemical parameter during said inert change. In one embodiment said normalization is by a division with the value of the parameter A0 of the fitted function of formula I. In one embodiment a normalized difference function, as shown in the following formula II, is used:

$$yII = \left(x - \left(\frac{1}{2}P1 \cdot \left(1 + \operatorname{erf}\left(\frac{x-m}{s \cdot \sqrt{2}}\right)\right) + A0\right)\right) \Big/ A0.$$

Thus, in one embodiment step b) reads: determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use and determining therewith also the parameters of a normalized difference function of formula II, and step c) reads: determining the difference between the experimental data of the inert change of the

physicochemical parameter of the at least second use and the function of formula II with the parameters determined in step b). In another embodiment said normalization is in the step of calculating the difference between the maximum value and the minimum value of the difference determined between the experimental data and the function of formula I fitted to said experimental data by normalizing said difference by dividing said difference with the maximum value of the experimental data of said physicochemical parameter during said inert change.

An exemplary difference function for a chromatogram for an inert change with no reduction in separation efficacy and/or packing quality is shown in FIG. **3** and for a chromatogram for an inert change with a reduction in separation efficacy and/or packing quality is shown in FIG. **6**.

For the calculation of the absolute difference value the global maximum and the global minimum of the difference between the experimental data and the fitted experimental data calculated for each experimental data point is determined. The difference between this maximum value and this minimum value is calculated and provides a parameter with which the packing quality of the re-useable chromatography column packing can be determined. In one embodiment said difference is normalized by dividing the calculated difference values with the maximum value of the experimental data used for the calculation.

Depending on the polypeptide to be purified and its characteristics to be achieved threshold values for the absolute difference between the maximum value and the minimum value of the difference function can be given whereby the individual exceeding of each of the threshold values results in an action to be performed. In one embodiment said difference between the maximum value and the minimum value of the normalized difference function can be accepted and the packing can be used further if the absolute value of the difference calculated is less than 0.2 or 0.1 or 0.05. In one embodiment said difference between the maximum value and the minimum value of the normalized difference can be accepted but additional analyses and/or assessments have to be performed to ensure the specification conformity of the purified polypeptide if the absolute value is 0.05 or more but less than 0.2, or 0.1 or more but less than 0.2, or 0.1 or more but less than 0.15. In one embodiment said difference between the maximum value and the minimum value of the normalized difference cannot be accepted and the packing has to be changed/renewed if the absolute value is 0.2 or more, or 0.15 or more.

Therefore, another aspect as reported herein is a method for the chromatographic purification of a polypeptide, wherein at least one chromatography step is contained which employs a re-useable chromatography column packing, characterized in that said method comprises the following steps:

a) identifying and determining the experimental data of an inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing,

b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use,

c) determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b),

d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference,

wherein the function of formula I is

$$yl = \frac{1}{2}P1 \cdot \left(1 + \mathrm{erf}\left(\frac{x-m}{s \cdot \sqrt{2}}\right)\right) + A0,$$

with the amplitude P1, the starting value A0, the mean value m, the standard deviation s, and with

$$\mathrm{erf}(x) = \frac{2}{\sqrt{\pi}} \sum_{0}^{\infty} \frac{(-1)^n x^{2n+1}}{(2n+1)n!},$$

and

further using the re-useable chromatography column packing when the absolute value of the difference calculated in step d) is 0.05 or less, or

performing additional assessment and/or characterization of the purified polypeptide when the absolute value of the difference calculated in step d) is 0.05 or more but less than 0.2, or

changing the re-useable chromatography column packing when the absolute value of the difference calculated in step d) is 0.2 or more.

A "polypeptide" is a polymer consisting of amino acids joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 20 amino acid residues may be referred to as "peptide", whereas molecules consisting of two or more polypeptides or comprising one polypeptide of more than 100 amino acid residues may be referred to as "protein". A polypeptide may also comprise non-amino acid components, such as carbohydrate groups, metal ions, or carboxylic acid esters. The non-amino acid components may be added by the cell, in which the polypeptide is expressed, and may vary with the type of cell. Polypeptides are defined herein in terms of their amino acid backbone structure or the nucleic acid encoding the same. Additions such as carbohydrate groups are generally not specified, but may be present nonetheless.

In one embodiment said polypeptide is recombinantly produced. In another embodiment said polypeptide is an immunoglobulin or an immunoglobulin conjugate. The term "immunoglobulin" refers to a protein consisting of one or more polypeptide(s) substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the different constant region genes as well as the myriad immunoglobulin variable region genes. Immunoglobulins may exist in a variety of formats, including, for example, Fv, Fab, and $F(ab)_2$ as well as single chains (scFv) or diabodies (e.g. Huston, J. S., et al., Proc. Natl. Acad. Sci. USA 85 (1988) 5879-5883; Bird, R. E., et al., Science 242 (1988) 423-426; in general, Hood, L. E., et al., Immunology, Benjamin N.Y., 2nd edition (1984); and Hunkapiller, T. and Hood, L. E., Nature 323 (1986) 15-16). The term "immunoglobulin conjugate" denotes a polypeptide comprising at least one domain of an immunoglobulin heavy or light chain conjugated via a peptide bond to a further polypeptide. The further polypeptide is either a non-immunoglobulin peptide, such as a hormone, or growth receptor, or antifusogenic peptide, or complement factor, or the like, or an immunoglobulin fragment, such as Fv, Fab, and $F(ab)_2$ as well as single chain antibody (scFv) or diabody.

Methods for purifying polypeptides and immunoglobulins are well established and widespread used and are employed either alone or in combination. Such methods are, for example and in certain embodiments, affinity chromatogra-

phy using microbial-derived proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange chromatography), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and preparative electrophoretic methods (such as gel electrophoresis, capillary electrophoresis) (Vijayalakshmi, M. A., Appl. Biochem. Biotech. 75 (1998) 93-102). In one embodiment said chromatography column packing is a chromatography material selected from an affinity chromatography material, or an ion exchange chromatography material, or a thiophilic adsorption chromatography material, or a hydrophobic interaction chromatography material, or an aromatic adsorption chromatography material, or a metal chelate affinity chromatography material, or a size exclusion chromatography material.

In another embodiment the method as reported herein is used for the determination if the process hardware except the chromatography material has reduced separation efficacy.

The following examples and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. As the polypeptide erythropoietin was available in sufficient quantities in our laboratory at the time the invention was made the invention is exemplified with this polypeptide. This should not be understood as limitation but only as an example of the invention. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

DESCRIPTION OF THE FIGURES

FIG. **1** Experimental data for an exemplary inert change of the conductivity for a re-useable chromatography column packing with no reduced separation efficacy/packing quality (open circles); X-axis: time [min]; Y-axis: conductivity [mS/cm].

FIG. **2** Experimental data for an exemplary inert change of the conductivity for a re-useable chromatography column packing with no reduced separation efficacy/packing quality (open circles) and the fitted function according to a function of formula I; X-axis: time [min]; Y-axis: conductivity [mS/cm].

FIG. **3** Absolute difference between the experimental data for an exemplary inert change of the conductivity for a re-useable chromatography column packing with no reduced separation efficacy/packing quality (open circles) and the fitted function according to formula I; X-axis: time; Y-axis: difference.

FIG. **4** Experimental data for an exemplary inert change of the conductivity for a re-useable chromatography column packing with reduced separation efficacy/packing quality (open circles); X-axis: time [min]; Y-axis: conductivity [mS/cm].

FIG. **5** Experimental data for an exemplary inert change of the conductivity for a re-useable chromatography column packing with reduced separation efficacy/packing quality (open circles) and the fitted function according to a function of formula I; X-axis: time [min]; Y-axis: conductivity [mS/cm].

FIG. **6** Absolute difference between the experimental data for an exemplary inert change of the conductivity for a re-useable chromatography column packing with reduced separation efficacy/packing quality (open circles) and the fitted function according to a function of formula I; X-axis: time [min]; Y-axis: difference [mS/cm].

FIG. **7** Monitoring of column integrity with a method as reported herein over 50 chromatographic cycles using formula II.

FIG. **8** Monitoring of column integrity with a method as reported herein using formula II.

Full circles: parameter derived for regeneration of column without changes in column packing.

Open circles: parameter derived for regeneration of column with a cracked bed during regeneration cycle No. 9.

EXAMPLE 1

Fermentation and Purification of Erythropoietin

Erythropoietin can be produced and purified e.g. according to WO 01/87329.

The purification comprises some chromatography steps. One of these is a Blue Sepharose chromatography. Blue Sepharose consists of Sepharose beads to the surface of which the Cibacron blue dye is covalently bound. Since erythropoietin binds more strongly to Blue Sepharose than most non-proteinaceous contaminants, some proteinaceous impurities and PVA, erythropoietin can be enriched in this step. The elution of the Blue Sepharose column is performed by increasing the salt concentration as well as the pH. The column is filled with Blue Sepharose, regenerated with NaOH and equilibrated with equilibration buffer (sodium/calcium chloride and sodium acetate). The acidified and filtered fermenter supernatant is loaded. After completion of the loading, the column is washed first with a buffer similar to the equilibration buffer containing a higher sodium chloride concentration and consecutively with a TRIS-base buffer. The product is eluted with a TRIS-base buffer and collected in a single fraction in accordance with the master elution profile.

During the equilibration, separation, and regeneration step of the chromatography cycle the conductivity of the mobile phase at the outlet of the column is determined and recorded with a standard conductivity measuring device.

EXAMPLE 2

Change in Column Properties

The column can be monitored over the process continuously using the method as reported herein. Subtle changes become detectable independently of changes of other process parameters. In FIG. **7** a change in the column packing resulting in a change in the separation properties is shown. The change occurred after cycle **40**.

In the case of a column with a broken bed, the quality of the fit decreases dramatically, as seen in the derived parameter residuals difference as shown in cycle **9** of FIG. **8** (open circles).

The invention claimed is:

1. A method for determining whether a re-useable chromatography column packing, which is used at least for the second time in a purification step of a purification of a polypeptide, has reduced separation efficacy in said purification step of said purification of said polypeptide, wherein said method comprises the following steps:

a) identifying and determining the experimental data of an inert change of at least one physicochemical parameter

of a mobile phase passing through said re-useable chromatography column packing of the at least second use,

b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use,

c) determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b),

d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference,

e) determining reduced separation efficacy of said re-useable chromatography column packing when the absolute value of the difference calculated in step d) is more than 0.1,

wherein the function of formula I is

$$yI = \frac{1}{2} P1 \cdot \left(1 + \text{erf}\left(\frac{x-m}{s \cdot \sqrt{2}}\right)\right) + A0,$$

with the amplitude P1, the starting value A0, the mean value m, the standard deviation s, and with

$$\text{erf}(x) = \frac{2}{\sqrt{\pi}} \sum_{0}^{\infty} \frac{(-1)^n x^{2n+1}}{(2n+1)n!}.$$

2. The method according to claim 1, wherein said inert change is recorded during the purification by a standard conductivity measuring device.

3. The method according to claim 1 wherein said inert change is recorded during the purification by a standard adsorption measuring device.

4. The method according to claim 1, wherein said inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing is a change effected by the change of the concentration of a substance in the mobile phase that does not interact with the re-useable column packing.

5. The method according to claim 1, wherein said inert change is a change in conductivity, as measured by a standard conductivity measuring device.

6. The method according to claim 1, wherein said inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing is a change of from 100% of a solution containing a denaturing agent to 100% of a solution not containing said denaturing agent, or vice versa.

7. The method according to claim 6, wherein said denaturing agent is selected from sodium hydroxide, guanidinium chloride, urea or organic solvent.

8. The method according to claim 1, wherein said inert change is a sigmoid change.

9. The method according to claim 1, wherein said inert change is a change over time.

10. A method for the chromatographic purification of a polypeptide, wherein at least one chromatography step using a re-useable chromatography column packing is contained, wherein said method comprises the following steps:

a) identifying and determining the experimental data of an inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing of the at least second use,

b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use,

c) determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b),

d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference,

wherein the function of formula I is

$$yI = \frac{1}{2} P1 \cdot \left(1 + \text{erf}\left(\frac{x-m}{s \cdot \sqrt{2}}\right)\right) + A0,$$

with the amplitude P1, the starting value A0, the mean value m, the standard deviation s, and with

$$\text{erf}(x) = \frac{2}{\sqrt{\pi}} \sum_{0}^{\infty} \frac{(-1)^n x^{2n+1}}{(2n+1)n!},$$

and

further using the re-useable chromatography column packing when the absolute value of the difference calculated in step d) is 0.05 or less, or

performing an additional characterization and/or assessment of the purified polypeptide when the absolute value of the difference calculated in step d) is more than 0.05 but less than 0.2, or

changing the re-useable chromatography column packing when the absolute value of the difference calculated in step d) is 0.2 or more.

11. The method according to claim 10, wherein said inert change is recorded during the purification by a standard conductivity measuring device.

12. The method according to claim 10, wherein said inert change is recorded during the purification by a standard adsorption measuring device.

13. The method according to claim 10, wherein said inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing is a change effected by the change of the concentration of a substance in the mobile phase that does not interact with the re-useable column packing.

14. The method according to claim 10, wherein said inert change is a change in conductivity, as measured by a standard conductivity measuring device.

15. The method according to claim 10, wherein said inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing is a change of from 100% of a solution containing a denaturing agent to 100% of a solution not containing said denaturing agent, or vice versa.

16. The method according to claim 15, wherein said denaturing agent is selected from sodium hydroxide, guanidinium chloride, urea or organic solvent.

17. The method according to claim 10, wherein said inert change is a sigmoid change.

18. The method according to claim 10, wherein said inert change is a change over time.

* * * * *

EXHIBIT JJ

US008633302B2

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Hepbildikler et al.

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(54) **VARIABLE TANGENTIAL FLOW FILTRATION**

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None
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(56) **References Cited**

U.S. PATENT DOCUMENTS

5,490,937	A	2/1996	Van Reis
6,252,055	B1	6/2001	Relton
2004/0167320	A1	8/2004	Couto et al.
2006/0051347	A1	3/2006	Winter
2006/0182740	A1	8/2006	Yang et al.

FOREIGN PATENT DOCUMENTS

EP	0907378	4/1999
WO	97/45140	4/1997
WO	97/45140	12/1997
WO	2004/076695	9/2004
WO	2005091801	6/2005
WO	2006/031560	3/2006
WO	2006/091801 A2	8/2006

OTHER PUBLICATIONS

(Translation of Jap Off Act in Corres Jap Appl 2010516413 Jul. 24, 2012).
Van Reis et al: "Bioprocess membrane technology" Jour. of Membrane Science 297:1-2 (2007) 16-50 XP02208062.
Reis Van R et al: "Membrance separations in biotechnology" Current Opinion in Biotechnology 12:2 (2001) 208-211 XP002317550.
Low et al: "Future of antibody purification" Journal of Chromatography B: Biomedical Sciences & Applications, 848:1 (2007) 48-63 XP005922827.
Dosmar, M. et al, "Factors influencing Ultrafiltration Scale-Up" Bioprocess International, Informa Life Sciences Group,3:40-50 (2005) XP009090780.
Van Reis R et al, "High-performance tangential flow filtration using charged membranes" Journal of Membrane Science 159:1-2 (1999) 133-142 XP004169169.
Velez D et al: "Use of tangential flow filtration in perfusion propagation of hybridoma cells for production of monoclonal antibodies" Biotechnology and Bioengineering 33;7 (1989) 938-940 XP000005263.
Membrane Technology, "TFF cassettes offer high reliability and performance" 2006:11 (2006) p. 4, XP005772756.
Gerhart et al, Fundamentals of Fluid Mechanics (1992) 2nd Ed.
Ahrer, et al, Jour of Membrane Sci 274 (2006) 108-115.
Cheryan, M. Ultrafiltration and Microfiltration Handbook (1998).
Lou et al, "High Concentration UF/DF of a Monoclonal Antibody" Bioproces Technical vol. 4 (2006) 44-47.
Shire, S et al, Jour. of Pharmaceutical Sciences93:6 (2004) 1390-1402.
Uber, D C et al, Biotechniques 11:5 (1991) 642-647.
Millipore Technical Brief: Protein Concentration and Diafiltration by Tangential Flow Filtration, 2003.
The Communication of the notice of opposition by the European Patent Office, issued on Oct. 2, 2013, in the co-pending European Patent No. EP 2170949., pp. 29.
Rosenberg et al., "Ultrafiltration concentration of monoclonal antibody solutions: Development of an optimized method minimizing aggregation" Journal of Membrane Science 342(1-2):50-59.

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(57) **ABSTRACT**

The current invention reports a method for concentrating an immunoglobulin solution by tangential flow filtration wherein the transmembrane pressure and the cross-flow are variable.

13 Claims, 15 Drawing Sheets

VARIABLE TANGENTIAL FLOW FILTRATION

The current invention is in the field of protein concentration, to be more precise it relates to the use of tangential flow filtration (TFF) for immunoglobulin concentration.

BACKGROUND OF THE INVENTION

Proteins and especially immunoglobulins play an important role in today's medical portfolio. Expression systems for the production of recombinant polypeptides are well-known in the state of the art and are described by, e.g., Marino, M. H., Biopharm. 2 (1989) 18-33; Goeddel, D. V., et al., Methods Enzymol. 185 (1990) 3-7; Wurm, F., and Bernard, A., Curr. Opin. Biotechnol. 10 (1999) 156-159. Polypeptides for use in pharmaceutical applications are mainly produced in mammalian cells such as CHO cells, NSO cells, Sp2/0 cells, COS cells, HEK cells, BHK cells, PER.C6® cells, and the like.

For human application every pharmaceutical substance has to meet distinct criteria. To ensure the safety of biopharmaceutical agents to humans, for example, nucleic acids, viruses, and host cell proteins, which would cause severe harm, have to be removed. To meet the regulatory specification one or more purification steps have to follow the manufacturing process. Among other, purity, throughput, and yield play an important role in determining an appropriate purification process.

Due to their chemical and physical properties, such as molecular weight and domain architecture including secondary modifications, the downstream processing of immunoglobulins is very complicated. For example, are not only for formulated drugs but also for intermediates in downstream processing (DSP) concentrated solutions required to achieve low volumes for economic handling and application storage. Furthermore short concentration times are favored to ensure smooth processes and short operating times. In this context imperfect TFF processes especially after final purification steps can cause sustained damage even affecting drug product. The correlation between shear stress and aggregation in tangential flow concentration processes for monoclonal antibody (mAb) intermediate solutions was investigated by Ahrer, K., et al. (J. Membr. Sci. 274 (2006) 108-115). The influence of concentration time and selected flow and pressure on process performance and aggregation status was monitored (see e.g. Dosmar, M., et al., Bioprocess Int. 3 (2005) 40-50; Luo, R., et al., Bioprocess Int. 4 (2006) 44-46).

Mahler, H.-C., et al. (Eur. J. Pharmaceut. Biopharmaceut. 59 (2005) 407-417) reported the induction and analysis of aggregates in a liquid IgG1-antibody formulation formed by different agitation stress methods. In U.S. Pat. No. 6,252,055 a concentrated monoclonal antibody preparation is reported. A method for producing a concentrated antibody preparation is reported in US 2006/0182740. A combined process including an ultrafiltration, a diafiltration, and a second ultrafiltration sequence is reported in US 2006/0051347. In EP 0 907 378 is reported a process for concentrating an antibody preparation using a cross-flow ultrafiltration with a fixed recirculation rate of 250 ml/min. Methods for tangential flow filtration and an apparatus therefore is reported in US 2004/0167320. In WO 97/45140 a concentrated antibody solution is reported.

SUMMARY OF THE INVENTION

The current invention provides a method for the concentration of solutions containing recombinantly produced immunoglobulins.

In more detail, one aspect of the current invention is a method for concentrating an immunoglobulin solution by tangential flow filtration wherein the transmembrane pressure and the cross-flow, which are applied, are variable with

a) a transmembrane pressure of from 1.4 bar to 1.6 bar and a cross-flow of from 75 ml/min. to 90 ml/min. in a concentration range up to 30 mg immunoglobulin per ml of solution to be concentrated,
b) a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 140 ml/min. to 160 ml/min. in a concentration range of from 15 mg/ml up to 55 mg/ml, and
c) a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 120 ml/min. to 140 ml/min. in a concentration range of more than 45 mg/ml.

In one embodiment the concentration range in step c) is of from 50 mg/ml up to 275 mg/ml. In a preferred embodiment the concentration range in step c) is of from 50 mg/ml up to 180 mg/ml. In a more preferred embodiment the concentration range in step c) is of from 50 mg/ml up to 130 mg/ml. In another embodiment the transmembrane pressure and cross-flow are 1.5 bar and 80 ml/min. in step a), 0.85 bar and 150 ml/min. in step b), and/or 0.85 bar and 130 ml/min. in step c). In another embodiment the immunoglobulin solution is a buffered, aqueous immunoglobulin solution.

Another aspect of the current invention is a method for producing a heterologous immunoglobulin comprising the following steps in this order:

a) providing a recombinant mammalian cell comprising one or more nucleic acids encoding a heterologous immunoglobulin,
b) cultivating the cell of step a) under conditions suitable for the expression of the heterologous immunoglobulin,
c) recovering the heterologous immunoglobulin from the recombinant mammalian cell or the culture medium,
d) concentrating the obtained aqueous, buffered solution comprising the heterologous immunoglobulin using a tangential flow filtration with variable transmembrane pressure and cross flow.

In one embodiment step d) of the method comprises concentrating the obtained aqueous, buffered solution using a tangential flow filtration with variable transmembrane pressure and cross flow with

i) a transmembrane pressure of from 1.4 bar to 1.6 bar and a cross-flow of from 75 ml/min. to 90 ml/min. in a concentration range up to 30 mg immunoglobulin per ml of solution to be concentrated,
ii) a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 140 ml/min. to 160 ml/min. in a concentration range of from 15 mg/ml up to 55 mg/ml, and
iii) a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 120 ml/min. to 140 ml/min. in a concentration range of more than 45 mg/ml.

In another embodiment the method comprises prior to step d) or after step d) the following step:

e) purifying the aqueous, buffered solution containing the heterologous immunoglobulin.

In another embodiment the heterologous immunoglobulin is a complete immunoglobulin, or an immunoglobulin fragment, or an immunoglobulin conjugate. In one embodiment the mammalian cell is a CHO cell, a BHK cell, a NSO cell, a Sp2/0 cell, a COS cell, a HEK cell, or a PER.C6® cell.

DETAILED DESCRIPTION OF THE INVENTION

The current invention reports a method for concentrating immunoglobulin solutions to a concentration of more than

100 mg/ml. It has been surprisingly found that with a method according to the invention this can be achieved with low aggregate formation and in short time.

The terms "tangential flow filtration" or "TFF", which are used interchangeably within the current invention, denote a filtration process wherein a solution containing a polypeptide to be concentrated flows along, i.e. tangential, to the surface of a filtration membrane. The filtration membrane has a pore size with a certain cut off value. In one embodiment the cut off value is in the range of 20 kDa to 50 kDa, preferably of 30 kDa. This filtration process is a kind of an ultrafiltration process. The term "cross-flow" denotes the flow of the solution to be concentrated tangential to the membrane (retentate flow). The term "flux" or "permeate flow", which can be used interchangeably within the current invention, denotes the flow of fluid across the membrane, i.e. through the pores of the membrane. That is, it denotes the volumetric rate of flow of the permeate through the membrane. A flow is usually given in terms of volume per unit membrane area per unit time as liters/m^2/h (LMH). In one embodiment the cross-flow is characterized in that the cross-flow is in ml/min for a membrane area of 0.02 m^2. In another embodiment the cross flow is in the individual steps 240 l/m^2/h, 450 l/m^2/h, and 390 l/m^2/h. The permeate comprises the solvent of the solution to be concentrated as well as molecules with a molecular weight below the cut off value of the employed membrane but not the heterologous immunoglobulin. The terms "transmembrane pressure" or "TMP", which can be used interchangeably within the current invention, denote the pressure which is applied to drive the solvent and components smaller than the cut off value of the filtration membrane through the pores of the filtration membrane. The transmembrane pressure is an average pressure of the inlet, outlet and permeate and can be calculated as:

$$TMP = \frac{(p_{in} + p_{out})}{2} - p_{permeate}$$

The term "immunoglobulin" refers to a protein consisting of one or more polypeptide(s) substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the different constant region genes as well as the myriad immunoglobulin variable region genes. Immunoglobulins may exist in a variety of formats, including, for example, Fv, Fab, and $F(ab)_2$ as well as single chains (scFv) or diabodies (e.g. Huston, J. S., et al., Proc. Natl. Acad. Sci. USA 85 (1988) 5879-5883; Bird, R. E., et al., Science 242 (1988) 423-426; in general, Hood et al., Immunology, Benjamin N.Y., 2nd edition (1984); and Hunkapiller, T. and Hood, L., Nature 323 (1986) 15-16).

The term "complete immunoglobulin" denotes an immunoglobulin which comprises two so called light immunoglobulin chain polypeptides (light chain) and two so called heavy immunoglobulin chain polypeptides (heavy chain). Each of the heavy and light immunoglobulin chain polypeptides of a complete immunoglobulin contains a variable domain (variable region) (generally the amino terminal portion of the polypeptide chain) comprising binding regions that are able to interact with an antigen. Each of the heavy and light immunoglobulin chain polypeptides of a complete immunoglobulin also comprises a constant region (generally the carboxyl terminal portion). The constant region of the heavy chain mediates the binding of the antibody i) to cells bearing a Fc gamma receptor (FcγR), such as phagocytic cells, or ii) to cells bearing the neonatal Fc receptor (FcRn) also known as Brambell receptor. It also mediates the binding to some factors including factors of the classical complement system such as component (C1q). The variable domain of an immunoglobulin's light or heavy chain in turn comprises different segments, i.e. four framework regions (FR) and three hypervariable regions (CDR).

The term "immunoglobulin fragment" denotes a polypeptide comprising at least one domain of the variable domain, the C_H1 domain, the hinge-region, the C_H2 domain, the C_H3 domain, the C_H4 domain of a heavy chain, the variable domain or the C_L domain of a light chain. Also comprised are derivatives and variants thereof. For example, a variable domain, in which one or more amino acids or amino acid regions are deleted, may be present.

The term "immunoglobulin conjugate" denotes a polypeptide comprising at least one domain of an immunoglobulin heavy or light chain conjugated via a peptide bond to a further polypeptide. The further polypeptide is a non-immunoglobulin peptide, such as a hormone, or growth receptor, or antifusogenic peptide, or complement factor, or the like.

General chromatographic methods and their use are known to a person skilled in the art. See for example, Chromatography, 5th edition, Part A: Fundamentals and Techniques, Heftmann, E. (ed.), Elsevier Science Publishing Company, New York, (1992); Advanced Chromatographic and Electromigration Methods in Biosciences, Deyl, Z. (ed.), Elsevier Science BV, Amsterdam, The Netherlands, (1998); Chromatography Today, Poole, C. F., and Poole, S. K., Elsevier Science Publishing Company, New York, (1991); Scopes, Protein Purification: Principles and Practice (1982); Sambrook, J., et al. (ed.), Molecular Cloning: A Laboratory Manual, second edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; or Current Protocols in Molecular Biology, Ausubel, F. M., et al. (eds), John Wiley & Sons, Inc., New York.

For the purification of recombinantly produced heterologous immunoglobulins often a combination of different column chromatography steps is employed. Generally a protein A affinity chromatography is followed by one or two additional separation steps. The final purification step is a so called "polishing step" for the removal of trace impurities and contaminants like aggregated immunoglobulins, residual HCP (host cell protein), DNA (host cell nucleic acid), viruses, or endotoxins. For this polishing step often an anion exchange material in a flow-through mode is used.

Different methods are well established and widespread used for protein recovery and purification, such as affinity chromatography with microbial proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and electrophoretical methods (such as gel electrophoresis, capillary electrophoresis) (Vijayalakshmi, M. A., Appl. Biochem. Biotech. 75 (1998) 93-102).

The term "heterologous immunoglobulin" denotes an immunoglobulin which is not naturally produced by a mammalian cell. The immunoglobulin produced according to the method of the invention is produced by recombinant means. Such methods are widely known in the state of the art and comprise protein expression in eukaryotic cells with subsequent recovery and isolation of the heterologous immunoglo-

bulin, and usually purification to a pharmaceutically acceptable purity. For the production, i.e. expression, of an immunoglobulin a nucleic acid encoding the light chain and a nucleic acid encoding the heavy chain are inserted each into an expression cassette by standard methods. Nucleic acids encoding immunoglobulin light and heavy chains are readily isolated and sequenced using conventional procedures. Hybridoma cells can serve as a source of such nucleic acids. The expression cassettes may be inserted into an expression plasmid(s), which is (are) then transfected into the host cell, which does not otherwise produce immunoglobulins. Expression is performed in appropriate prokaryotic or eukaryotic host cells and the immunoglobulin is recovered from the cells after lysis or from the culture supernatant.

The term "immunoglobulin solution" as used within the current application denotes an aqueous, buffered solution containing a complete immunoglobulin, an immunoglobulin fragment, or an immunoglobulin conjugate. This solution may be, e.g., a culture supernatant, or a column chromatography eluate, or a polished immunoglobulin solution.

"Heterologous DNA" or "heterologous polypeptide" refers to a DNA molecule or a polypeptide, or a population of DNA molecules or a population of polypeptides, that do not exist naturally within a given host cell. DNA molecules heterologous to a particular host cell may contain DNA derived from the host cell species (i.e. endogenous DNA) so long as that host DNA is combined with non-host DNA (i.e. exogenous DNA). For example, a DNA molecule containing a non-host DNA segment encoding a polypeptide operably linked to a host DNA segment comprising a promoter is considered to be a heterologous DNA molecule. Conversely, a heterologous DNA molecule can comprise an endogenous structural gene operably linked with an exogenous promoter. A peptide or polypeptide encoded by a non-host DNA molecule is a "heterologous" peptide or polypeptide.

The term "under conditions suitable for the expression of the heterologous immunoglobulin" denotes conditions which are used for the cultivation of a mammalian cell expressing an immunoglobulin and which are known to or can easily be determined by a person skilled in the art. It is also known to a person skilled in the art that these conditions may vary depending on the type of mammalian cell cultivated and type of immunoglobulin expressed. In general the mammalian cell is cultivated at a temperature of from 20° C. to 40° C., and for a period of time sufficient to allow effective protein production of the immunoglobulin, e.g. of from 4 to 28 days.

The term "recombinant mammalian cell" refers to a cell into which a nucleic acid, e.g. encoding a heterologous polypeptide, can be or is introduced/transfected. The term "cell" includes cells which are used for the expression of nucleic acids. In one embodiment the mammalian cell is a CHO cell (e.g. CHO K1, CHO DG44), or a BHK cell, or a NSO cell, or a SP2/0 cell, or a HEK 293 cell, or a HEK 293 EBNA cell, or a PER.C6® cell, or a COS cells. In a preferred embodiment the mammalian cell is a CHO cell, or a BHK cell, or HEK cell, or Sp2/0 cell, or a PER.C6® cell. As used herein, the expression "cell" includes the subject cell and its progeny. Thus, the term "recombinant cell" include the primary transfected cell and cultures including the progeny cells derived there from without regard to the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological activity as the originally transformed cell are included.

The term "buffered" as used within this application denotes a solution in which changes of pH due to the addition or

release of acidic or basic substances is leveled by a buffer substance. Any buffer substance resulting in such an effect can be used. In one embodiment pharmaceutically acceptable buffer substances are used, such as e.g. phosphoric acid or salts thereof, acetic acid or salts thereof, citric acid or salts thereof, morpholine or salts thereof, 2-(N-morpholino) ethanesulfonic acid or salts thereof, Histidine or salts thereof, Glycine or salts thereof, or tris (hydroxymethyl) aminomethane (TRIS) or salts thereof. In a preferred embodiment the buffer substance is phosphoric acid or salts thereof, acetic acid or salts thereof, or citric acid or salts thereof, or histidine or salts thereof. Optionally the buffered solution may comprise an additional salt, such as e.g. sodium chloride, and/or sodium sulphate, and/or potassium chloride, and/or potassium sulfate, and/or sodium citrate, and/or potassium citrate. In one embodiment of the invention the pH value of the buffered aqueous solution is of from pH 3.0 to pH 10.0, preferably of from pH 3.0 to pH 7.0, more preferred of from pH 4.0 to pH 6.0, and most preferred of from pH 4.5 to pH 5.5.

It has now surprisingly been found that with a tangential flow filtration (TFF) method according to the current invention in which the transmembrane pressure and cross-flow are variable during the filtration process and are adapted depending on the actual concentration of the immunoglobulin in the solution to be concentrated a concentrated immunoglobulin solution with low aggregate formation can be obtained in a short time. That is, it has surprisingly been found that aggregate formation during tangential flow filtration is low if a TFF method according to the invention is applied, i.e. a method in which during the filtration process the transmembrane pressure is changed and adapted according to the actual concentration of the antibody solution. The method according to the invention is a variable method compared to constant methods as known from the art, i.e. to methods in which the transmembrane pressure is adopted prior to the filtration process and is held constant during the entire tangential flow filtration process.

The current invention comprises a method for concentrating an immunoglobulin solution by tangential flow filtration wherein the transmembrane pressure and the cross-flow, which are applied, are variable and changed during the filtration process depending on the immunoglobulin concentration in the concentrated immunoglobulin solution, whereby

a) a transmembrane pressure of from 1.4 bar to 1.6 bar and a cross-flow of from 75 ml/min. to 90 ml/min. is applied in a concentration range up to 30 mg immunoglobulin per ml of the solution to be concentrated,

b) a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 140 ml/min. to 160 ml/min. is applied in a concentration range of from 15 mg/ml up to 55 mg/ml, and

c) a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 120 ml/min. to 140 ml/min. is applied in a concentration range of more than 45 mg/ml.

A correlation between shear stress in TFF and aggregate formation exists. To evaluate the effect the flow-induced shear stress τ_w on the surface of the used membrane was calculated with the following formula

$$\tau_w = \frac{d_H(\Delta p)}{4L} \text{ wherein } d_H \text{ is } d_H = 4\frac{ab}{2(a+b)}$$

based on Gerhart, et al. (Fundamentals of Fluid Mechanics, Addison-Wesley Publishing Company (1993)) and on Cheryan, et al. (Ultrafiltration and Microfiltration Handbook,

second edition CRC Press LLC (1998)). In this formula d_H is the hydraulic diameter, a the width, b the height, and L the length of the flow channel. Further, $\Delta p=p_i-p_o$, with p_i is the applied inlet pressure, and p_o is the outlet pressure. In one example a Hydrosart™ membrane (Sartocon Slice 200 Hydrosart™ of Sartorius AG, Göttingen, Germany) consisting of regenerated cellulose, with a nominal molecular weight cut off of 30 kDa and a membrane area of 0.02 m^2 was employed. For the used membrane cassette a hydraulic diameter of 1.08 mm was calculated. The membrane was at first operated with a standard TFF method, i.e. without a change of transmembrane pressure and cross-flow during the concentration process. Three different constant methods with preset, constant Δp and a preset, constant transmembrane pressure (TMP) of 0.6 bar were analyzed.

TABLE 1

Overview of applied pressure differences and the corresponding shear stress.

Δp [bar]	τ_w [Pa]
1.2	216
1.8	324
3.0	541

By observing flux versus ascending protein concentration there is no significant difference in the curves for processes at different Δp. But for the 3 bar mode a lower end concentration due to a high inlet pressure was observed. Compared to a concentration mode performed under a lower constant cross-flow (CF; 90 ml/min.) and a lower mean Δp (about 0.9 bar) a higher Δp of 1.2-1.8 bar contributes to an improved flux performance over time and a higher end concentration (TMP always 0.6 bar).

Comparing turbidity, light obscuration (LO), and dynamic light scattering (DLS) data before and after the concentration process showed that enhanced aggregate formation was found with increased shear stress (FIG. **2**).

A TFF method has been developed with comparable overall process time compared to the stressing high inlet pressure mode (Δp=3 bar), based on TMP/CF-scouting experiments (see e.g. Luo, R., et al., Bioprocess Int. 4 (2006) 44-54). The method according to the invention has been developed to improve flux performance over time with reduced immunoglobulin aggregate formation, i.e. to combine a low aggregate formation and a short overall concentration time. During the developing of the method according to the invention a TMP and CF scouting was performed depending on the prevailing immunoglobulin concentration in the immunoglobulin solution to be concentrated. An method with adapted TMP and CF depending on the best flux profile at a given concentration was found. Without the disadvantage of high inlet pressure at the final stage of the concentration (see e.g. Dosmar, M., et al., Bioprocess Int. 3 (2005) 40-50) the method according to the invention showed a low aggregation burden in turbidity, LO, and DLS data for produced concentrates (see FIGS. **3** and **4**). In addition a higher end concentration was achieved with the method according to the invention.

In the method according to the invention the transmembrane pressure and cross-flow are varied with respect to the actual concentration of the concentrated immunoglobulin solution. In one embodiment the method according to the invention is a variable tangential flow filtration method wherein the actual concentration of the immunoglobulin in the solution to be concentrated determines the applied transmembrane pressure and cross-flow. Thus, the transmembrane pressure and cross-flow are adjusted depending on the actual concentration of the immunoglobulin in order to reduce the stress applied and, thus, to reduce the formation of aggregated immunoglobulin molecules and to provide a short overall concentration time.

In the method according to the invention three concentration ranges are defined. The first actual concentration range of the solution to be concentrated is from 0 mg/ml to 30 mg/ml, the second actual concentration range is from 15 mg/ml to 55 mg/ml, and the third actual concentration range is from 45 mg/ml to 180 mg/ml. As it can be seen these concentration ranges are overlapping ranges. It has been found that in the overlapping concentration ranges of from 15 mg/ml to 30 mg/ml and of from 45 mg/ml to 55 mg/ml different values for the transmembrane pressure and the cross-flow can be used in the method according to the invention. In these overlapping concentration ranges any of the two TMP and CF settings can be applied without a notable effect on aggregated formation or process time.

Thus, in one embodiment of the method according to the invention the conditions from a) to b) and from b) to c) can be changed at any concentration value in the overlapping concentration ranges.

In one embodiment the concentration range in step c) is of from 50 mg/ml up to 275 mg/ml. In another embodiment the transmembrane pressure and cross-flow is 1.5 bar and 80 ml/min. in step a), 0.85 bar and 150 ml/min. in step b), and/or 0.85 bar and 130 ml/min. in step c). In another embodiment is the immunoglobulin solution a buffered, aqueous immunoglobulin solution. In one embodiment the concentration range is in step a) of 5 to 25 mg/ml, in step b) of from 25 to 50 mg/ml, and in step c) of from 50 to 140 mg/ml.

Another aspect of the current invention is a method for producing a heterologous immunoglobulin comprising the following steps in the following order:

a) providing a recombinant mammalian cell comprising one or more nucleic acids encoding a heterologous immunoglobulin,
b) cultivating the mammalian cell under conditions suitable for the expression of the heterologous immunoglobulin,
c) recovering the heterologous immunoglobulin from the recombinant mammalian cell or the culture medium as aqueous, buffered solution,
d) concentrating the obtained aqueous, buffered solution comprising the heterologous immunoglobulin using a tangential flow filtration with variable, immunoglobulin concentration dependent transmembrane pressure and cross-flow.

In one embodiment of the production method according to the invention comprises step d) concentrating the obtained aqueous, buffered solution using a tangential flow filtration with variable, immunoglobulin concentration dependent transmembrane pressure and cross-flow with

i) a transmembrane pressure of from 1.4 bar to 1.6 bar and a cross-flow of from 75 ml/min. to 90 ml/min. in a concentration range up to 30 mg immunoglobulin per ml of solution to be concentrated,
ii) a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 140 ml/min. to 160 ml/min. in a concentration range of from 15 mg/ml up to 55 mg/ml, and
iii) a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 120 ml/min. to 140 ml/min. in a concentration range of more than 45 mg/ml.

In another embodiment the method comprises prior to, i.e. before, or after step d) the following step:

e) purifying the aqueous, buffered solution containing the heterologous immunoglobulin.

The purification in step e) can be by different methods and techniques, such as a chromatography step, or a combination of different or similar chromatographic steps, or precipitation, or salting out, or ultrafiltration, or diafiltration, or lyophilization, or buffer change, or combinations thereof, or the like.

In another embodiment the heterologous immunoglobulin is a complete immunoglobulin, or an immunoglobulin fragment, or an immunoglobulin conjugate. In one embodiment the mammalian cell is a CHO cell, a BHK cell, a NSO cell, a Sp2/0 cell, a COS cell, a HEK cell, or a PER.C6® cell. In a preferred embodiment the mammalian cell is a CHO cell, or a BHK cell, or a HEK cell, or a Sp2/0 cell, or a PER.C6® cell.

The following examples and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

An anti-IL-IR antibody (see e.g. WO 2005/023872) and an anti-P-selectin antibody (see e.g. WO 2005/100402) were available in sufficient quantities in our laboratories at the time of the invention and therefore the current invention is exemplified with these two immunoglobulins. Likewise the invention is in general practicable with any immunoglobulin. This exemplified description is done only by way of example and not by way of limitation of the invention.

DESCRIPTION OF THE FIGURES

FIG. **1** Flux versus protein concentration of an anti-IL-IR antibody solution before flushing of the membrane for different constant Δp modes and a concentration method under constant CF of 90 ml/min. 1: constant method Δp=1.2 bar, 2: constant method Δp=1.8 bar, 3: constant method Δp=3.0 bar, 4: constant method CF 90 ml/min.

FIG. **2** Number of particles before and after concentration of an anti-IL-IR antibody solution with constant method. 1: before concentration, 2: τ_w=216, 3: τ_w=324, 4: τ_w=541.

FIG. **3** Comparison of number of particles of an anti-IL-IR antibody solution before and after concentration with different methods. 1: before concentration, 2: variable method according to the invention, 3: constant method CF 90 ml/min., 4: τ_w=541.

FIG. **4** Flux versus protein concentration of an anti-IL-IR antibody solution. 1: constant method CF=90 ml/min., 2: τ_w=541, 3: variable method according to the invention.

FIG. **5** Transmembrane flux versus transmembrane pressure of an anti-IL-IR antibody solution at a protein concentration of 5.3 mg/ml for cross-flows of 50 ml/min. (filled circles), 80 ml/min. (filled triangles), and 130 ml/min. (filled squares).

FIG. **6** Transmembrane flux versus transmembrane pressure of an anti-IL-IR antibody solution at a protein concentration of 45 mg/ml for cross-flows of 80 ml/min. (filled circles), 130 ml/min. (filled triangles), and 150 ml/min. (filled squares).

FIG. **7** Transmembrane flux versus transmembrane pressure of an anti-IL-IR antibody solution at a protein concentration of 90 mg/ml for cross-flows of 50 ml/min. (filled circles), 80 ml/min. (filled triangles), and 130 ml/min. (filled squares).

FIG. **8** Transmembrane flux versus transmembrane pressure of an anti-IL-IR antibody solution at a protein concentration of 180 mg/ml for cross-flows of 50 ml/min. (filled circles), 80 ml/min. (filled triangles), and 130 ml/min. (filled squares).

FIG. **9** Particle analysis of the concentrate of an anti-IL-IR antibody solution in citrate buffer obtained by different methods. 1: before concentration, 2: variable method according to the invention, 3: constant method CF 90 ml/min., 4: constant method Δp=1.8 bar, 5: constant method Δp=3.0 bar.

FIG. **10** Dynamic light scattering analysis of the concentrate of an anti-IL-IR antibody solution in citrate buffer obtained by different methods. Filled rhombus: before concentration, filled square: variable method according to the invention, filled triangle: constant method CF 90 ml/min., filled circles: constant method ΔP=1.8 bar.

FIG. **11** Turbidity measurement of concentrate of an anti-IL-IR antibody solution in citrate buffer obtained by different methods. 1: before concentration, 2: variable method according to the invention, 3: constant method CF=90 ml/min., 4: constant method Δp=1.8 bar, 5: constant method Δp=3.0 bar.

FIG. **12** Turbidity measurement of concentrate of an anti-IL-IR antibody solution obtained with the method according to the invention and a constant method employing different buffers. 1: before concentration in citrate buffer, 2: variable method according to the invention with citrate buffer, 3: constant method Δp=3.0 bar with citrate buffer, 4: before concentration in histidine buffer, 5: variable method according to the invention with histidine buffer, 6: constant method Δp=3.0 bar with histidine buffer.

FIG. **13** Dynamic light scattering analysis of the concentrate obtained by different methods and obtained in different buffer: a) anti-IL-IR antibody in citrate buffer (filled rhombus: before concentration, filled square: after concentration with variable method according to the invention, filled triangle: constant method Δp=1.8 bar), b) anti-IL-IR antibody in histidine buffer (filled rhombus: before concentration, filled square: after concentration with variable method according to the invention, filled triangle: constant method Δp=3.0 bar).

FIG. **14** Turbidity measurement (a) and dynamic light scattering (b) results of the concentration of an anti-P-selectin antibody in histidine buffer. 1: before concentration (filled rhombus), 2: variable method according to the invention (filled square), 3: constant method Δp=3.0 bar (filled triangle).

FIG. **15** Effect of concentration mode on filterability of concentrated immunoglobulin solutions.

EXAMPLE 1

Analytical Methods

a) Turbidity Measurement.

The photometric absorbance was determined at 350 nm and 550 nm, where no intrinsic chromophores in the antibody solution absorb (UV-VIS spectrophotometer Evolution 500, Thermo Fisher Scientific, Waltham, USA). The samples were measured undiluted. As a reference medium the appropriate buffer solution was used. Every measurement was conducted three times.

b) Size-Exclusion-HPLC.

The chromatography was conducted with a Tosoh Haas TSK 3000 SWXL column on an ASI-100 HPLC system (Dionex, Idstein, Germany). The elution peaks were monitored at 280 nm by a UV diode array detector (Dionex). After dissolution of the concentrated samples to 1 mg/ml the column was washed with a buffer consisting of 200 mM potassium dihydrogen phosphate and 250 mM potassium chloride pH 7.0 until a stable baseline was achieved. The analyzing

runs were performed under isocratic conditions using a flow rate of 0.5 ml/min. over 30 minutes at room temperature. The chromatograms were integrated manually with Chromeleon (Dionex, Idstein, Germany). Aggregation in % was determined by comparing the area under the curve (AUC) of high molecular weight forms with the AUC of the monomer peak.

c) Light Obscuration.

To monitor the particle burden in a range of 1-200 μm a SVSS-C particle analyzer was used (PAMAS Partikelmess- and Analysesysteme, Rutesheim, Germany). The system was calibrated according to the requirements of US Pharmacopeia Vol. 24, <788>, with near-monosize polystyrene spheres. Three measurements of a volume of 0.5 ml with a pre-flushing volume of 0.5 ml were performed. Results were calculated as mean value and referred to a sample volume of 1.0 ml. The number of particles counted was within the sensor's concentration limit.

d) Dynamic Light Scattering (DLS).

DLS is a non-invasive technique for measuring particle size, typically in the sub-micron size range. In the current invention the Zetasizer Nano S apparatus (Malvern Instruments, Worcestershire, UK) with a temperature controlled quartz cuvette (25° C.) was used for monitoring a size range between 1 nm and 6 μm. The intensity of the back scattered laser light was detected at an angle of 173°. The intensity fluctuates at a rate that is dependent upon the particle diffusion speed, which in turn is governed by particle size. Particle size data can therefore be generated from an analysis of the fluctuation in scattered light intensity (Dahneke, B. E. (ed), Measurement of Suspended Particles by Quasielectric Light Scattering, Wiley Inc. (1983); Pecora, R., Dynamic Light Scattering: Application of Photon Correlation Spectroscopy, Plenum Press (1985)). The size distribution by intensity was calculated using the multiple narrow mode of the DTS software (Malvern). Experiments were conducted with undiluted samples.

e) Fourier-Transformed Infrared Spectroscopy.

The FT-IR spectra of the undiluted protein solutions were recorded by using a Tensor 27 spectrometer (Bruker Optik, Ettlingen, Germany) with a flow-through transmission cell (AquaSpec) connected to a thermostat. For each spectrum a 120-scan interferogram was collected at a single-beam mode with a 4 cm^{-1} resolution. As reference media the appropriate permeate was used. The collected interferogram of the protein and the buffer system were Fourier transformed. Further, the spectrum of the protein was corrected for the spectrum of the corresponding buffer system.

EXAMPLE 2

Determination of TMP and CF Conditions

A conditioned and filtered citrate-buffered aqueous solution (pH 5.5) of an anti-IL-IR antibody was concentrated twenty fold up to 100 mg/ml by use of an automated TFF system ÄKTAcrossflow™ (GE Healthcare, Amersham Bioscience AB, Uppsala, Sweden) by employing a scaleable flat sheet cassette (Sartorius, Göttingen, Germany) with a Hydrosart™ membrane of regenerated cellulose, with a nominal molecular weight cut-off of 30 kDa and a membrane area of 0.02 m^2. Different concentration programs generated with the UNICORN software controlling ÄKTAcrossflow™ were performed. Total membrane loading was about 400 g/m^2.

Flux and pressure profiles at four preset transmembrane pressures are determined at different immunoglobulin concentrations in the immunoglobulin solution to be concentrated with respect to different cross-flows. The TMP was set to 0.3 bar, 0.5 bar, 0.9 bar, or 2.0 bar. The cross-flows for each TMP and protein concentration were 50 ml/min, 80 ml/min, 130 ml/min. (not at 45 mg/ml protein concentration), and 150 ml/min. (only at 45 mg/ml protein concentration). The different protein concentrations were 5.3 mg/ml, 45 mg/ml, 90 mg/ml, and 180 mg/ml. The results are shown in FIGS. **5** to **8**.

It has been found during the concentration processes that a high feed flux and a high feed pressure result in a good transmembrane flux. But during the concentration process, especially at the end, a polarization layer is established resulting in a membrane overpressure and also a reduced (permeate) flux. It was also found that an increased feed pressure results in a higher flux and therefore a fast concentration process but this acceleration is accompanied by an increased aggregate formation (FIGS. **9** to **11**).

Taking into account the above the ranges and conditions for an improved method for immunoglobulin concentration were found to be:

- a transmembrane pressure of from 1.4 bar to 1.6 bar and a cross-flow of from 75 ml/min. to 90 ml/min. in a concentration range up to 30 mg immunoglobulin per ml of solution to be concentrated,
- a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 140 ml/min. to 160 ml/min. in a concentration range of from 15 mg/ml up to 55 mg/ml, and
- a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 120 ml/min. to 140 ml/min. in a concentration range of more than 45 mg/ml.

These parameters result in a method with a reduced aggregate formation and a short concentration time.

EXAMPLE 3

Comparison of the Variable Method According to the Invention to Constant Methods

The method according to the invention was compared to different constant parameter methods for the production of a concentrated immunoglobulin solution. The target concentration was set to 90 mg/ml. The tangential flow filtration was performed with the devices according to Example 2. The different parameters of the compared methods (methods 1 to 4 are constant methods, method 5 is the variable method according to the invention) were the following:

Method 1: transmembrane pressure=0.6 bar
 cross-flow=90 ml/min
 Δp=0.7 bar

Method 2: transmembrane pressure=0.6 bar
 Δp=1.2 bar

Method 3: transmembrane pressure=0.6 bar
 Δp=1.8 bar

Method 4: transmembrane pressure=0.6 bar
 Δp=3.0 bar

Method 5: a) transmembrane pressure=1.5 bar, Δp=0.5 bar,
 b) transmembrane pressure=0.85 bar, Δp=1.2 bar,
 c) transmembrane pressure=0.85 bar.

The different parameters and the time required to achieve a concentration of the immunoglobulin solution to an immunoglobulin concentration of 90 mg/ml are shown in Table 2.

TABLE 2

Comparison of the parameters for different concentrations methods.

Method	ΔP	TMP	Feed press	Ret press	Feed flow	Ret flow	Particles >1 μm/ml	time
1	0.7 bar	0.6 bar	1.2 bar	0.5 bar	100 ml/min	90 ml/min	7321820	149 min.
2	1.2 bar	0.6 bar	1.4 bar	0.2 bar	170 ml/min	160 ml/min	15403850	126 min.
3	1.8 bar	0.6 bar	2.1 bar	0.3 bar	230 ml/min	215 ml/min	16989540	125 min.
4	3.0 bar	0.6 bar	2.8 bar	0.2 bar	300 ml/min	280 ml/min	19415180	116 min.
5	0.5 bar	1.5 bar	2.0 bar	1.5 bar	100 ml/min	80 ml/min	12182240	118 min.
	1.2 bar	0.85 bar	1.7 bar	0.5 bar	165 ml/min	150 ml/min		
	—	0.85 bar	—	0.5 bar	135 ml/min	130 ml/min		

From the results of the different methods it can be seen that with method 5, i.e. with a variable method, compared to methods 2 to 4 a dramatically reduced aggregate formation can be obtained and thus an immunoglobulin concentrate with improved characteristics. Compared to method 1 a faster concentration process can be achieved.

EXAMPLE 4

Concentration of an Anti-IL-IR Antibody in Different Buffer Systems

A comparative concentration of an aqueous anti-IL-IR antibody solution buffer with citrate buffer or histidine buffer was performed with the device of Example 2 and the method according to the invention (method S of Example 3). The results are shown in FIGS. **12** and **13**. From FIG. **12** and FIG. **13**, respectively, can be seen that the employed buffer has no effect on the concentration process according to the invention.

EXAMPLE 5

Concentration of an Anti-P-Selectin Antibody

The concentration of an anti-P-selectin antibody was performed according to the method of Example 2 and the results are shown in FIG. **14**.

EXAMPLE 6

Filtration of Concentrated Solution

The concentrated solution obtained according to the method of Example 2 was filtrated after the tangential flow filtration with a pressure of 0.75 bar through a Durapore (PVDF, Millipore GmbH, Schwalbach, Germany) membrane (4.52 cm^2 filter area).

It has been found that the filterability of highly concentrated immunoglobulin solutions depends on the employed concentration method. It has further been found that the concentrated immunoglobulin solution obtained with the variable method according to the invention show a reduced decline in the filtration flow when compared with other fixed methods (FIG. **15**).

The invention claimed is:

1. A method for concentrating an immunoglobulin solution by tangential flow filtration, characterized in that the transmembrane pressure and the cross-flow are variable, and changed during the filtration process according to the concentration of the immunoglobulin to be concentrated, wherein

i) a transmembrane pressure of from 1.4 bar to 1.6 bar and a cross-flow of from 75 ml/min. to 90 ml/min. is applied in a concentration range up to 30 mg immunoglobulin per ml of solution to be concentrated,

ii) a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 140 ml/min. to 160 ml/min. is applied in a concentration range of from 15 mg/ml up to 55 mg/ml, and

iii) a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 120 ml/min. to 140 ml/min is applied in a concentration range of more than 45 mg/ml up to about 130 mg/ml.

2. The method of claim **1**, wherein the transmembrane pressure and cross-flow

in i) are 1.5 bar and 80 ml/min.,

in ii) are 0.85 bar and 150 ml/min., and;

in iii) are 0.85 bar and 130 ml/min.

3. The method of claim **1**, wherein the concentration range is

in i) of 5 to 25 mg/ml,

in ii) of from 25 to 50 mg/ml, and

in iii) from 50 to 140 mg/ml.

4. A method for producing a heterologous immunoglobulin comprising:

a) providing a recombinant mammalian cell comprising one or more nucleic acids encoding a heterologous immunoglobulin,

b) cultivating said cell under conditions suitable for the expression of the heterologous immunoglobulin,

c) recovering the heterologous immunoglobulin from the recombinant mammalian cell or the culture medium; and

d) concentrating the obtained aqueous, buffered solution comprising the heterologous immunoglobulin using a tangential flow filtration wherein the concentration of the heterologous immunoglobulin determines the transmembrane pressure and cross flow in said filtration and

wherein the variable transmembrane pressure and cross-flow of step d) are selected from the group consisting of

i) a transmembrane pressure of from 1.4 bar to 1.6 bar and a cross-flow of from 75 ml/min to 90 ml/min. in a concentration range up to 30 mg immunoglobulin per ml of solution to be concentrated,

ii) a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 140 ml/min. to 160 ml/min. in a concentration range of from 15 mg/ml up to 55 mg/ml,

iii) a transmembrane pressure of from 0.8 bar to 0.9 bar and a cross-flow of from 120 ml/min. to 140 ml/min. in a concentration range of more than 45 mg/ml up to about 130 mg/ml.

5. The method of claim **4**, further comprising prior to or after step d), the following:

e) purifying the aqueous buffered solution containing the heterologous immunoglobulin.

6. The method of claim **5**, wherein the heterologous immunoglobulin is a complete immunoglobulin, or an immunoglobulin fragment, or an immunoglobulin conjugate.

7. The method of claim **6**, characterized in that the mammalian cell is a CHO cell, a BHK cell, a HEK cell, or a Sp2/0 cell.

8. The method of claim **4**, wherein said tangential flow filtration employs a membrane with a cut off value in the range of from 20 to 50 kDa molecular weight.

9. The method of claim **4**, wherein said immunoglobulin solution has a pH value of from pH 3.0 to pH 10.0.

10. The method claim **9**, wherein said pH value is in the range of from pH 3.0 to pH 7.0.

11. The method of claim **4**, characterized in that said method is a variable tangential flow filtration method wherein the actual concentration of the immunoglobulin in the solution to be concentrated determinates the applied transmembrane pressure and cross-flow.

12. The method of claim **4**, characterized in that the transmembrane pressure and cross-flow can be changed at any concentration value in the overlapping concentration ranges.

13. The method of claim **4**, characterized in that the concentration range is

in i) of 5 to 25 mg/ml,

in ii) of from 25 to 50 mg/ml, and

in iii) of from 50 to 140 mg/ml.

* * * * *

Fig. 1

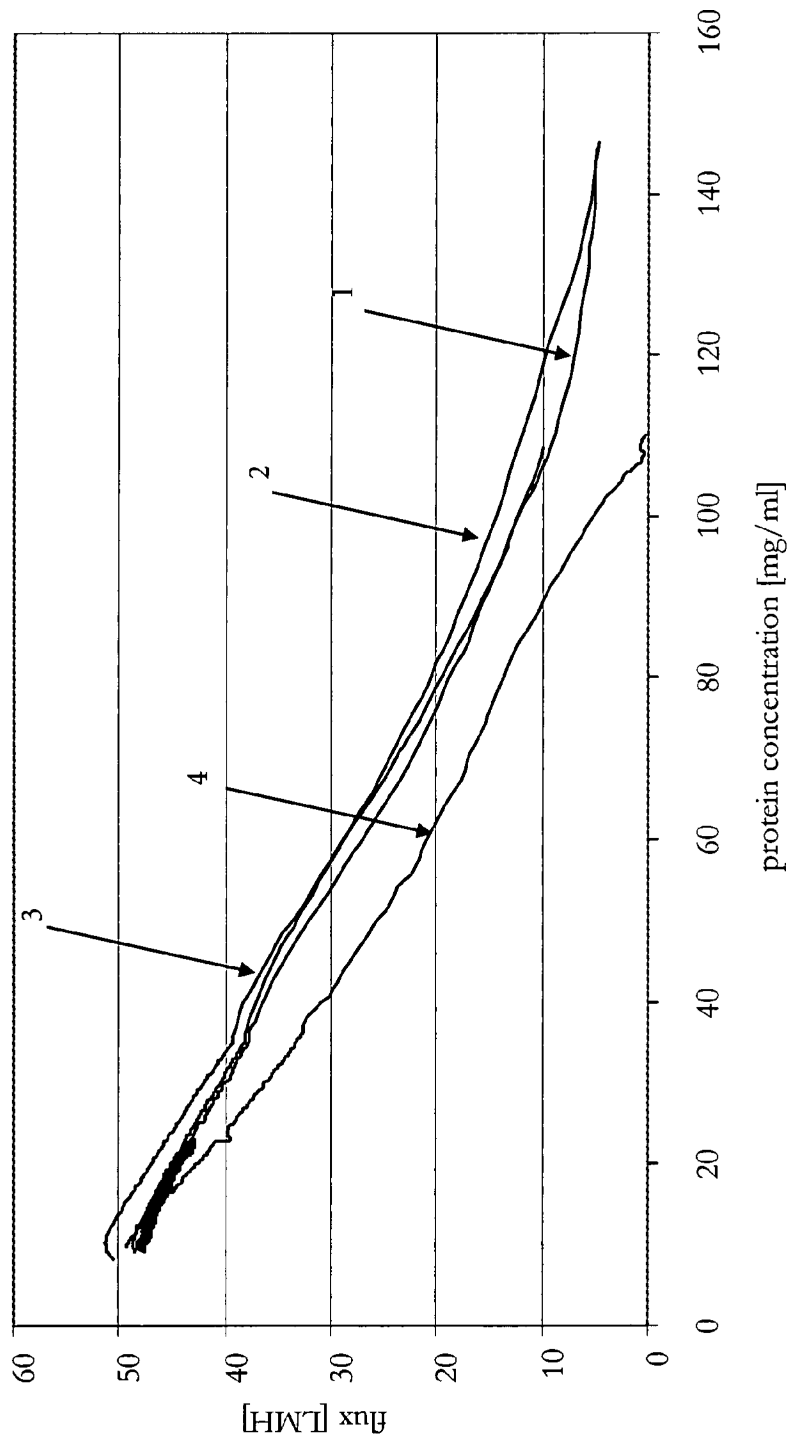

Fig. 2

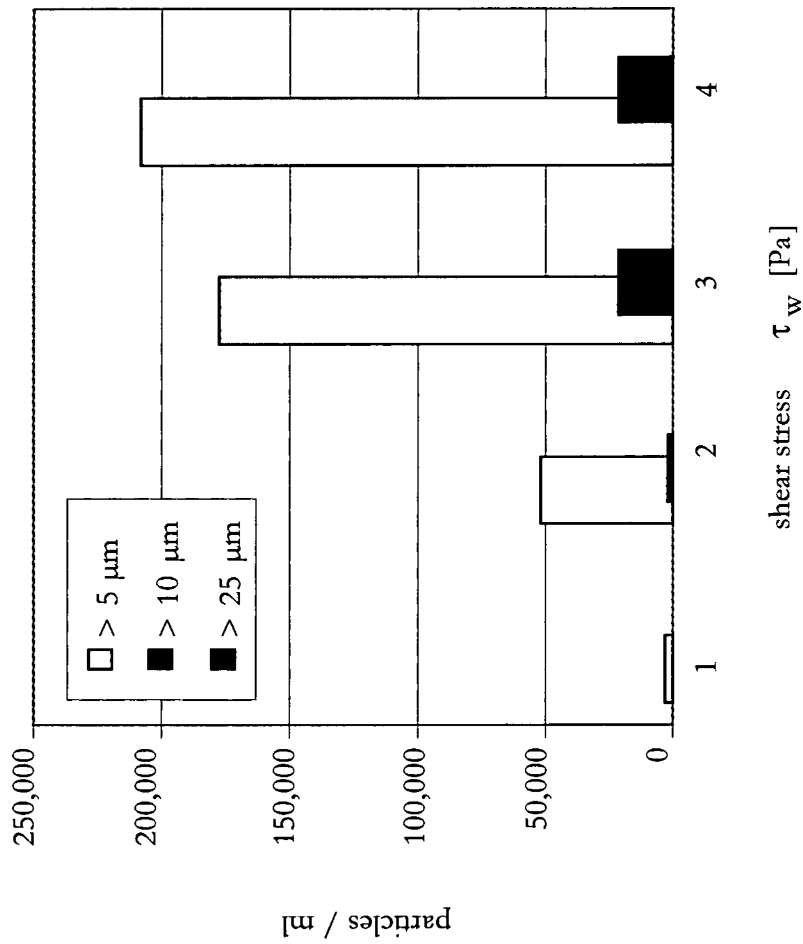

Fig. 3

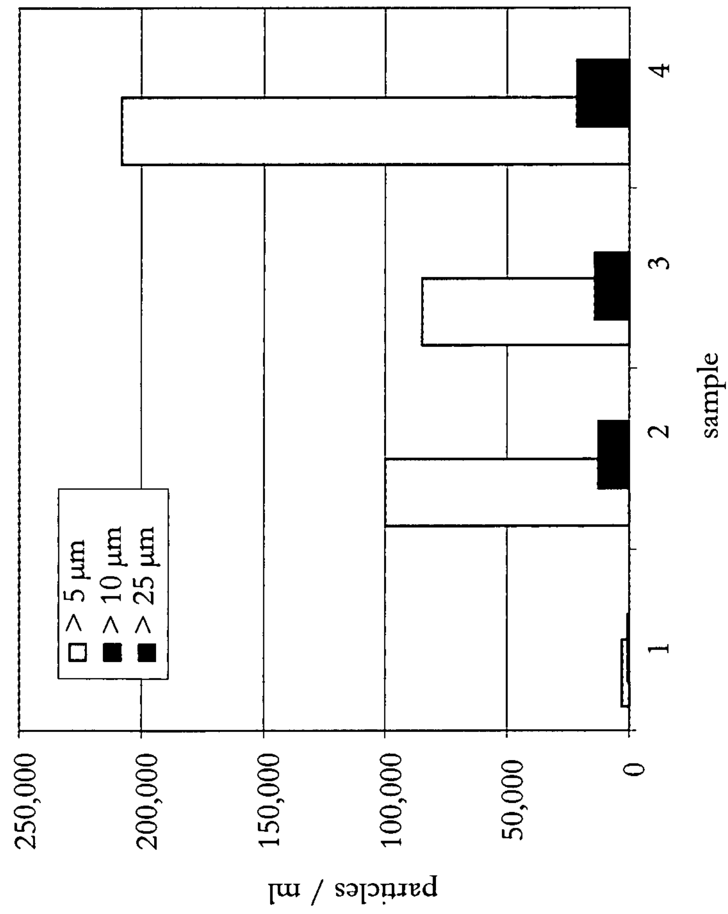

Fig. 4

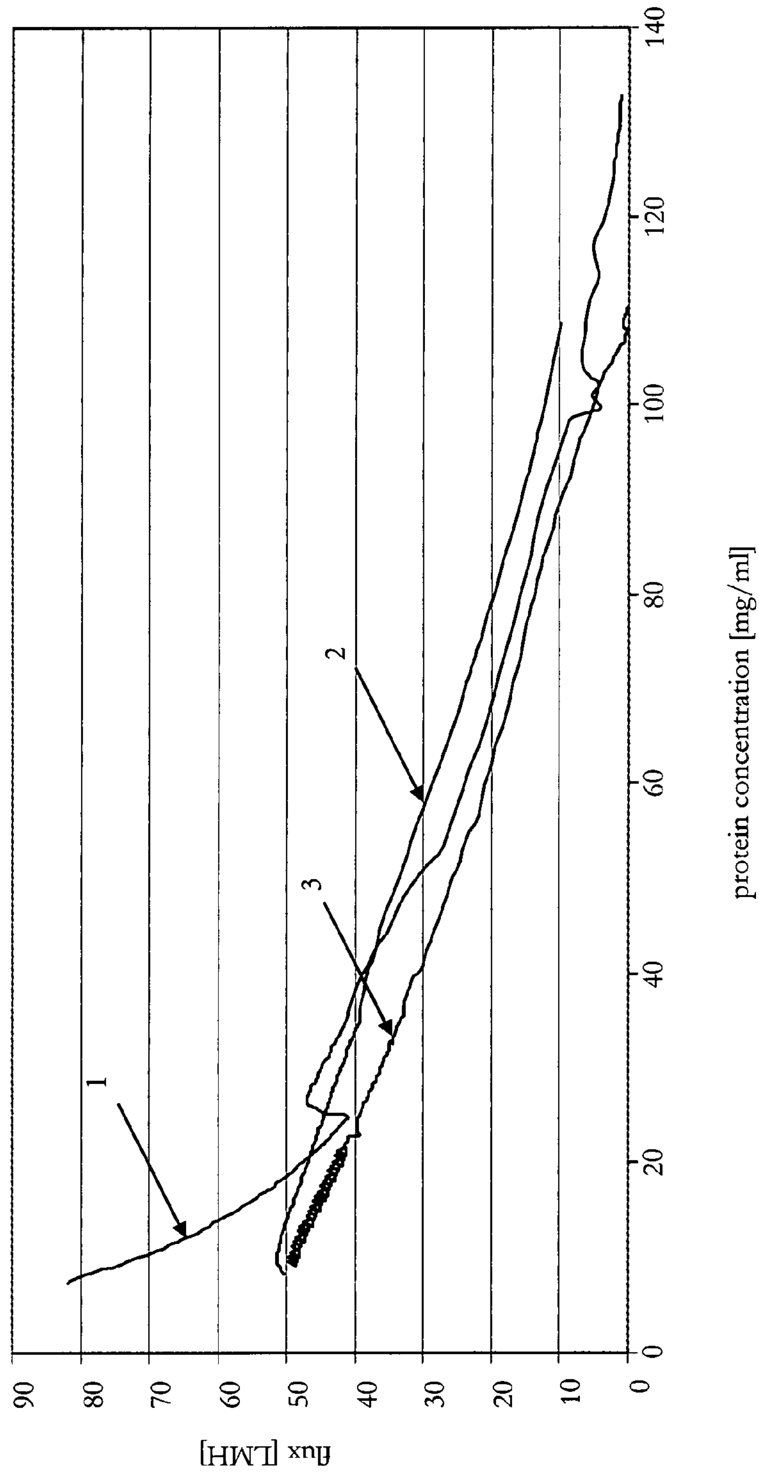

Fig. 5

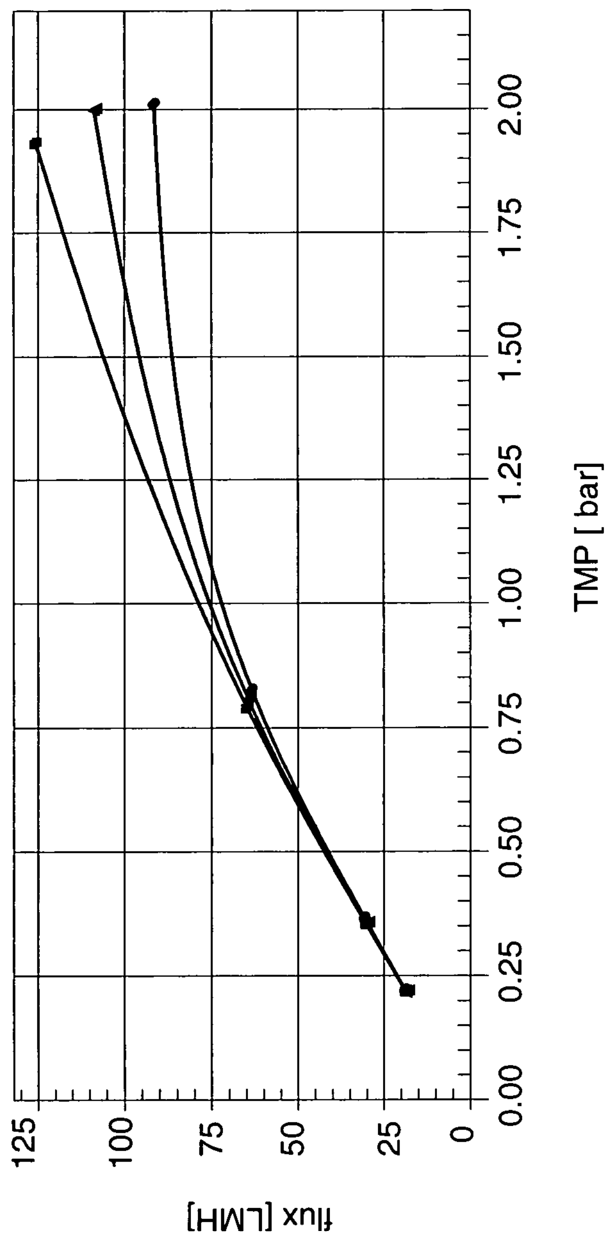

Fig. 6

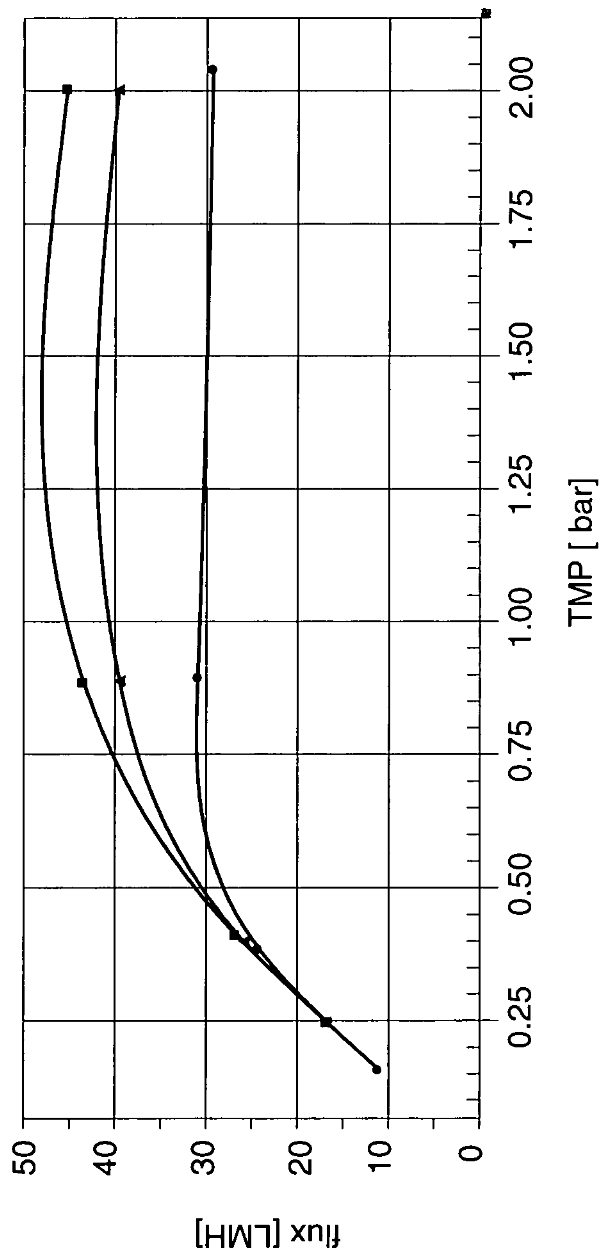

Fig. 7

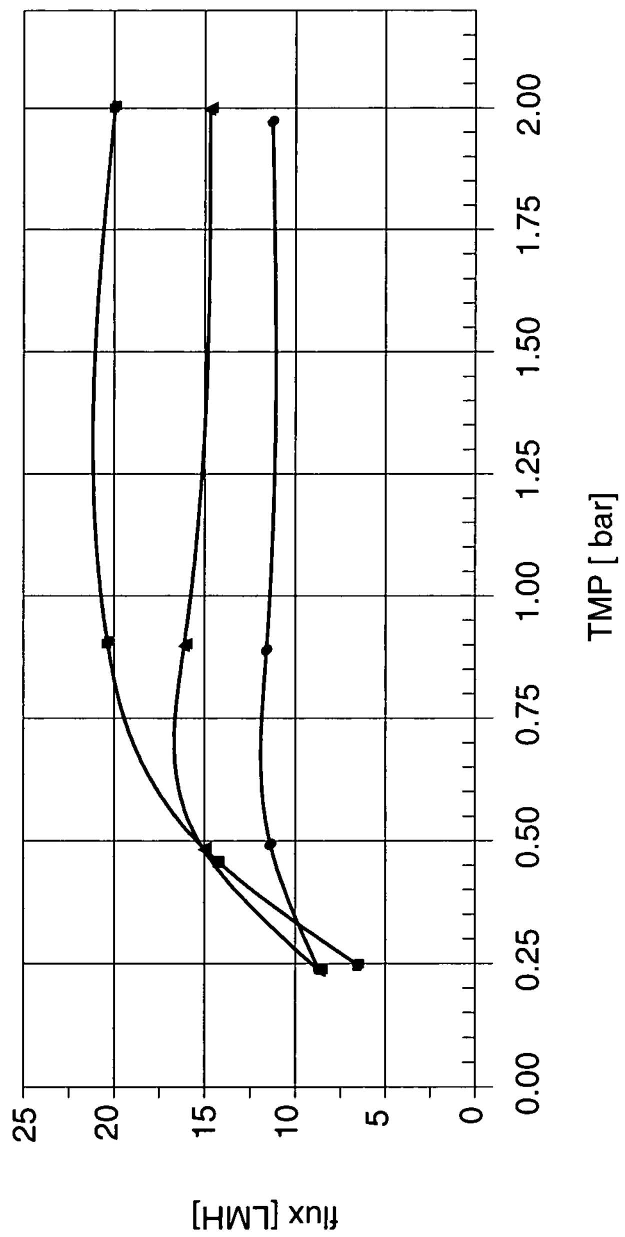

Fig. 8

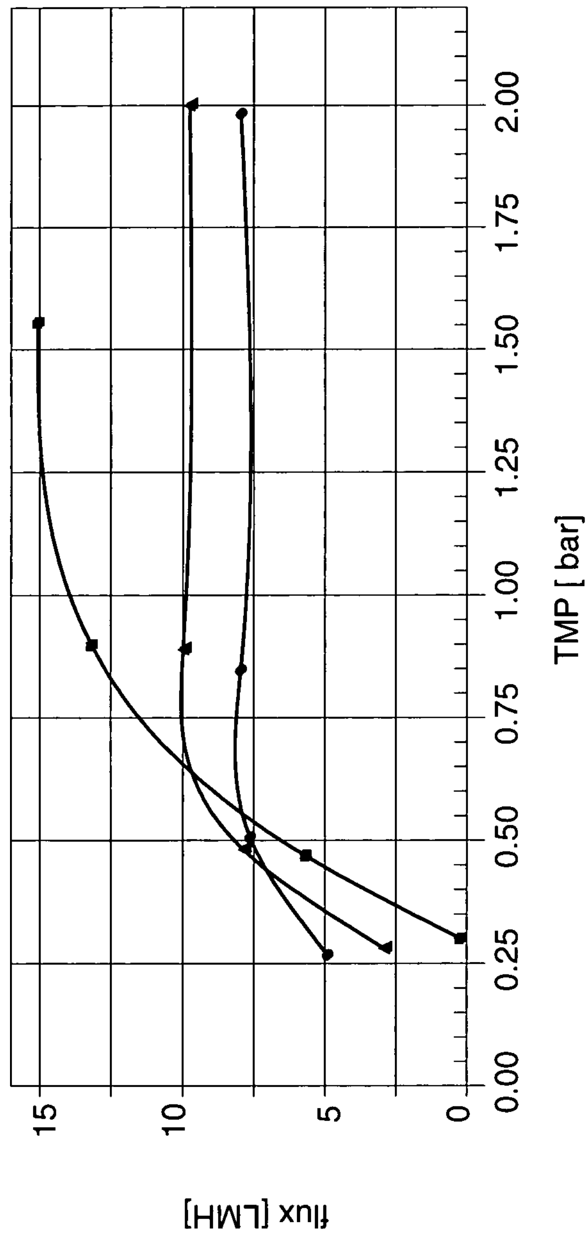

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Fig. 9

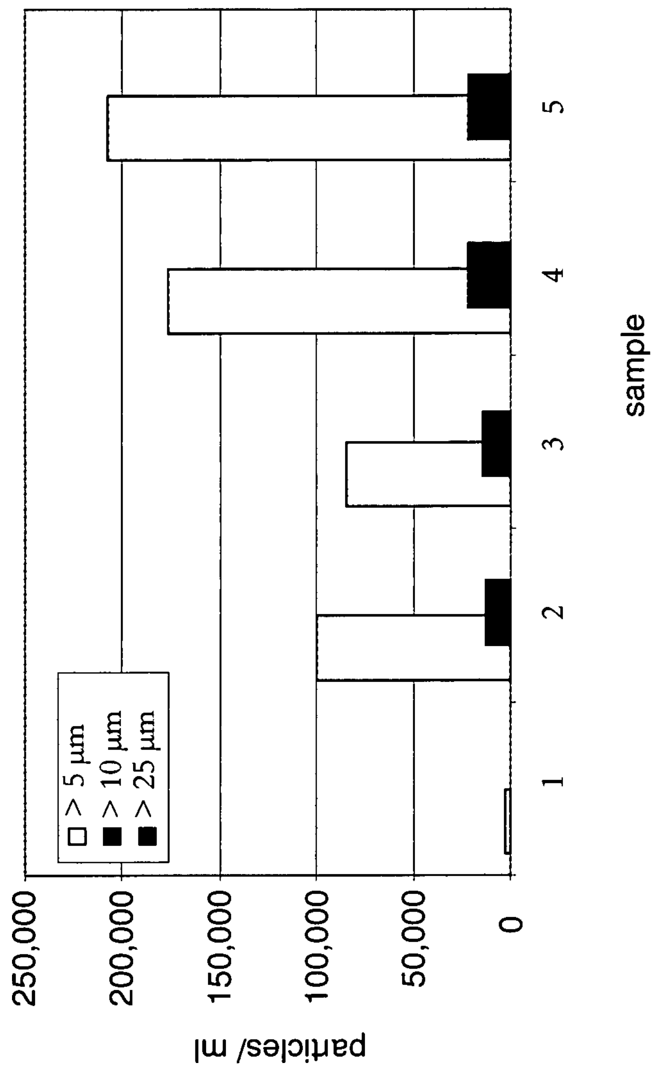

Fig. 10

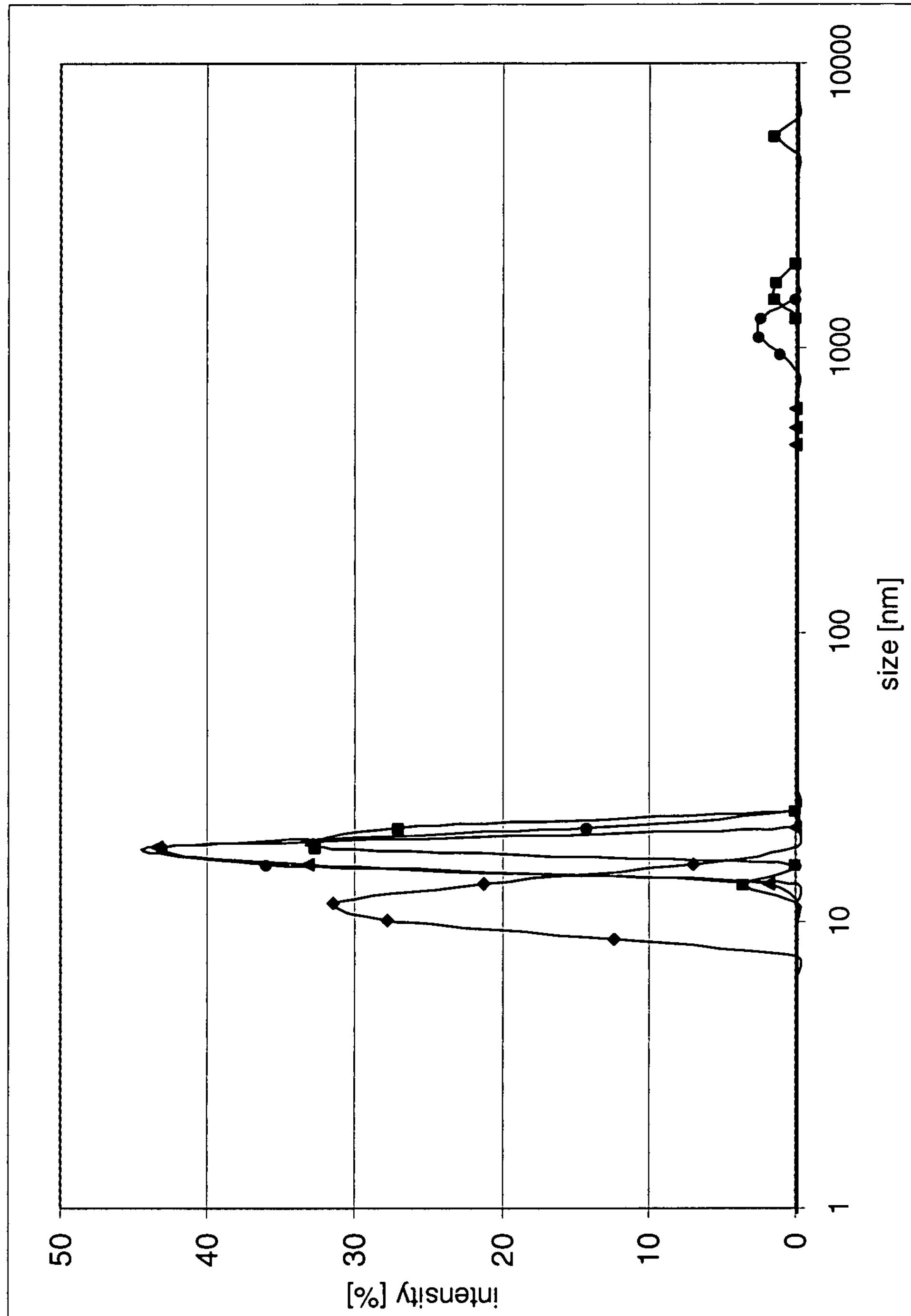

Fig. 11

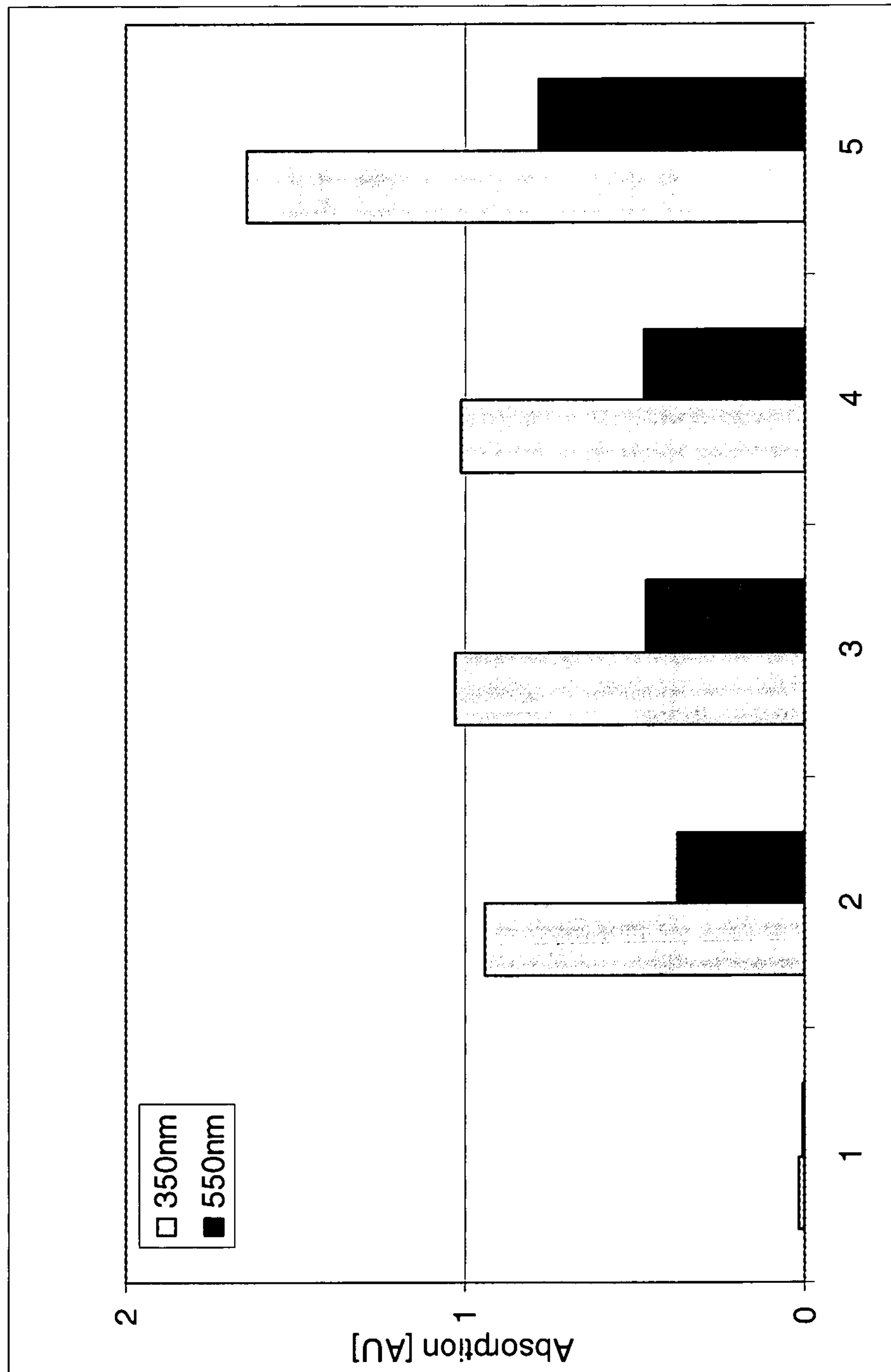

Fig. 12

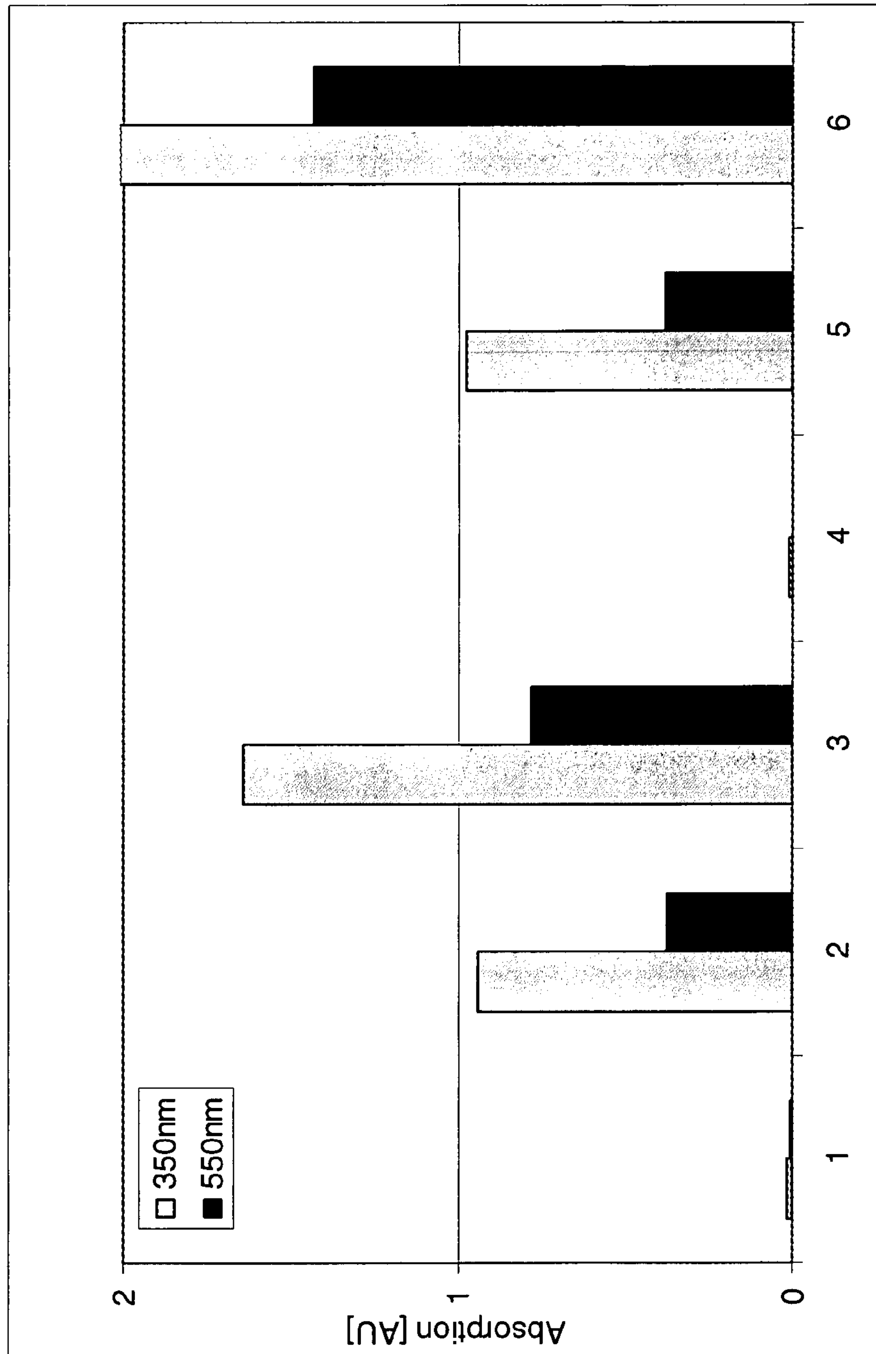

Fig. 13

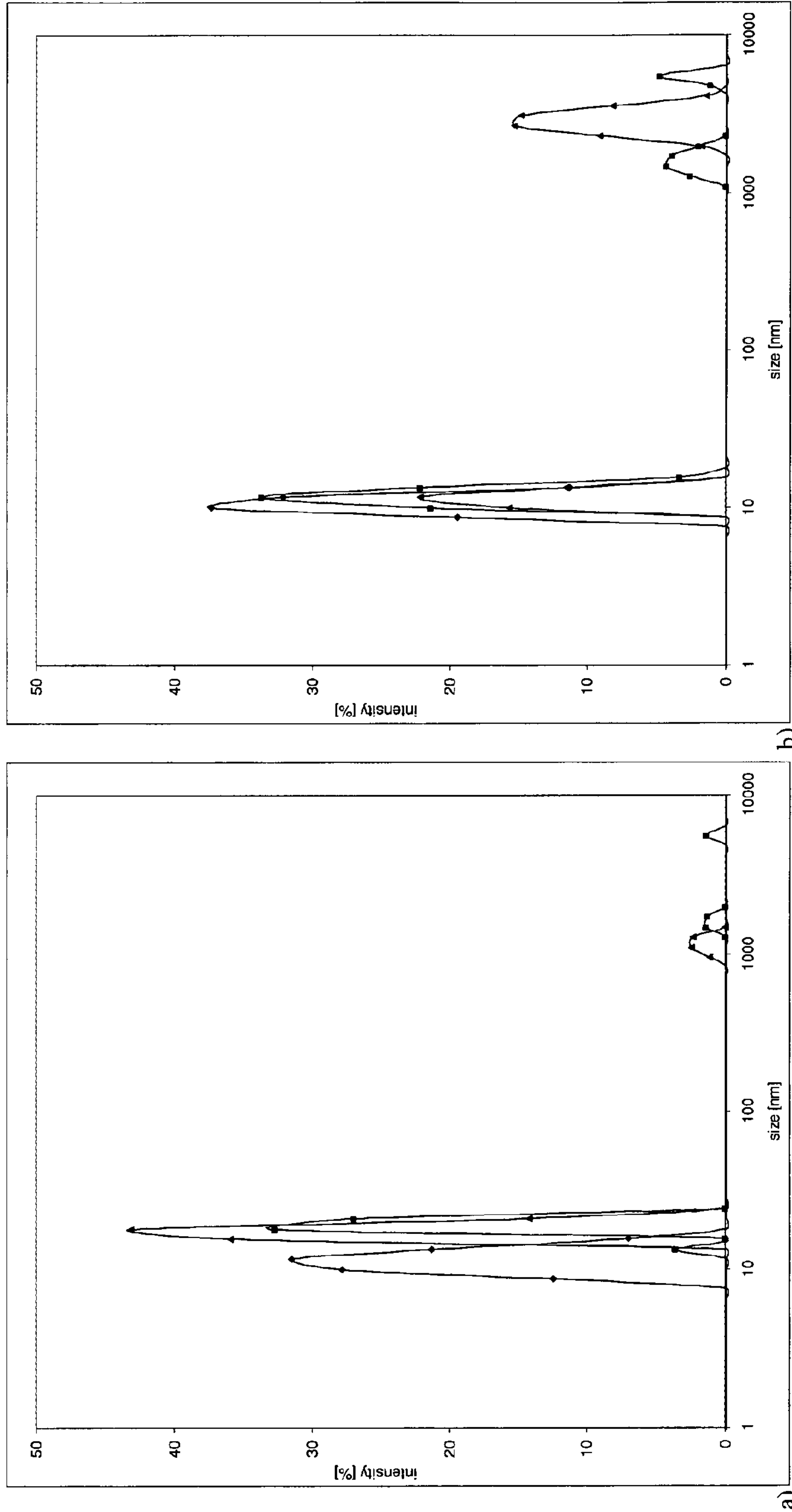

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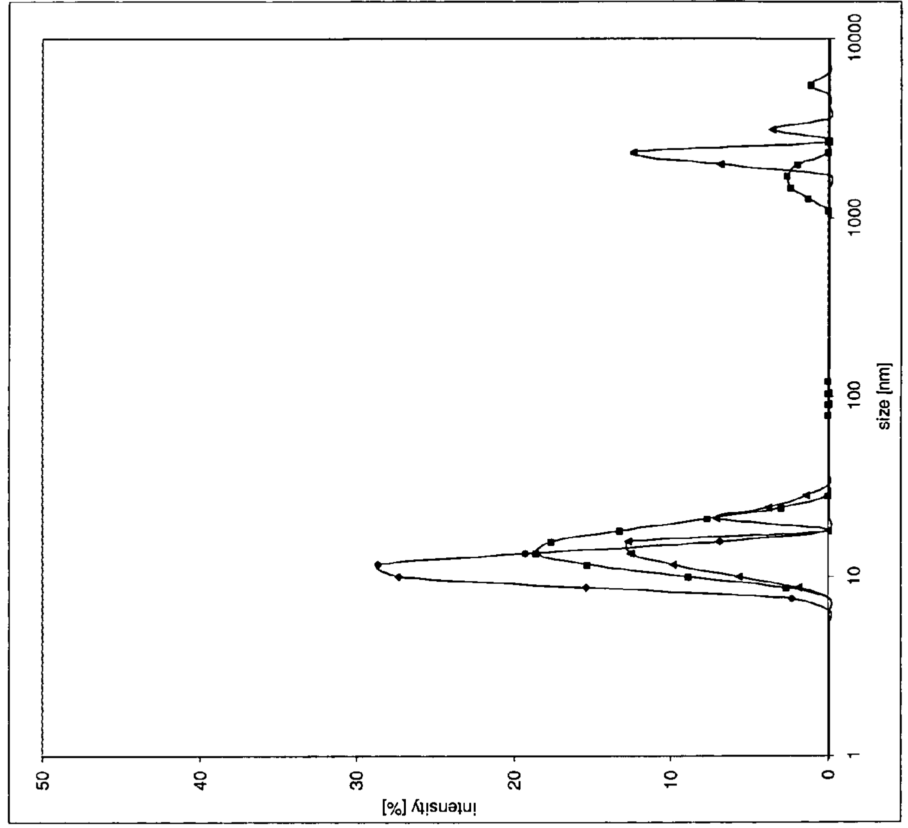
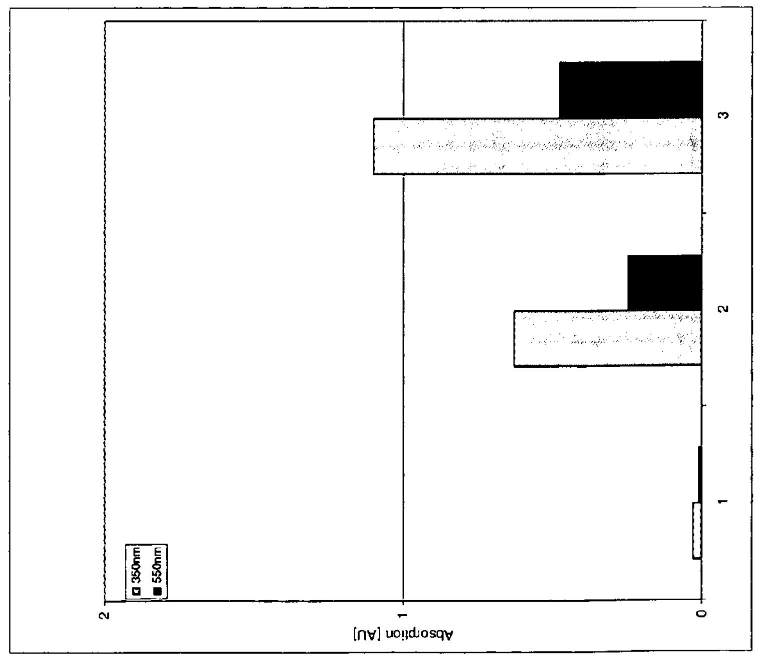
Fig. 14

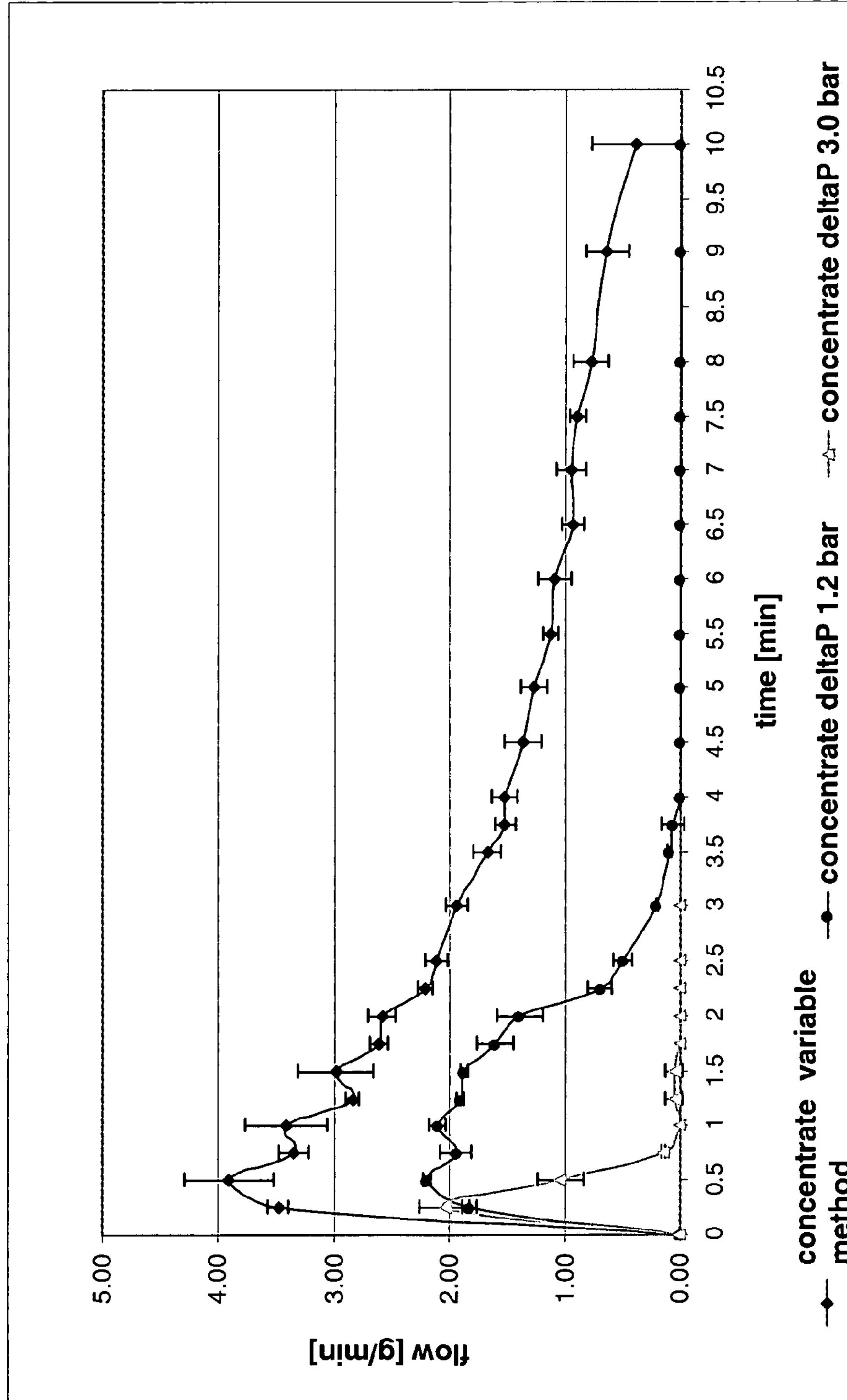


b)



a)

Fig. 15



UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,633,302 B2
APPLICATION NO. : 12/668661
DATED : January 21, 2014
INVENTOR(S) : Hepbildikler et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page:

The first or sole Notice should read --

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 738 days.

Signed and Sealed this
Twenty-second Day of September, 2015

Michelle K. Lee
Director of the United States Patent and Trademark Office

EXHIBIT KK

US008822655B2

(12) **United States Patent**
Hepbildikler et al.

(10) **Patent No.: US 8,822,655 B2**
(45) **Date of Patent: Sep. 2, 2014**

(54) **PRE-FILTRATION ADJUSTMENT OF BUFFER SOLUTES**

(75) Inventors: **Stefan Hepbildikler**, Munich (DE); **Wolfgang Kuhne**, Penzberg (DE); **Eva Rosenberg**, Munich (DE); **Gerhard Winter**, Penzberg (DE)

(73) Assignee: **Hoffmann-La Roche Inc.**, Nutley, NJ (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 355 days.

(21) Appl. No.: **13/395,893**

(22) PCT Filed: **Aug. 27, 2010**

(86) PCT No.: **PCT/EP2010/062554**

§ 371 (c)(1), (2), (4) Date: **May 16, 2012**

(87) PCT Pub. No.: **WO2011/039012**

PCT Pub. Date: **Apr. 7, 2011**

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(30) **Foreign Application Priority Data**

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(51) **Int. Cl.**

C07K 16/00	(2006.01)
A23J 1/00	(2006.01)
C07K 1/14	(2006.01)
C07K 1/34	(2006.01)
B01D 61/00	(2006.01)
B01D 61/14	(2006.01)
C07K 16/18	(2006.01)

(52) **U.S. Cl.**

CPC ... ***C07K 1/14*** (2013.01); ***C07K 1/34*** (2013.01); *B01D 61/00* (2013.01); *B01D 61/14* (2013.01); *C07K 16/18* (2013.01)

USPC **530/412**; 530/387.1; 530/414

(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,490,937	A	2/1996	van Reis
2005/0169925	A1	8/2005	Bardroff et al.
2005/0226876	A1	10/2005	Graus et al.

FOREIGN PATENT DOCUMENTS

WO	03/070760	8/2003
WO	2005/100402	10/2005
WO	2006/031560	3/2006
WO	2009/010269	1/2009

OTHER PUBLICATIONS

Faude, A. et al., Journal of Chromatography 1161:29-35 (2007).
Winzor, D et al., Anal. Biochem. 333:225-229 (2004).
Daugherty, A. et al., Advanced Drgu jDelivery Rev 58:686-706 (2006).
Saxena, A. et al., Advances in Collid and Interface Science 145:1-22 (2009).
Van Reis, et al., Journal of Membrane Science 129:19-29 (1997).
Capelle, M et al., Eur. Journal of Pharm. Biopharm 65:131-148 (2007).
Nakatsuka, S. et al., Journal of Membrane Science 69(SUPPL 189-211) (1992).
Mignard, D et al., Journal of Membrane Science 186:133-143 (2001).
(Written Opinion by Internat'l Search Authority in PCT/EP2010/ 062554 Jul. 29, 2011).
The Japanese Office Action, issued on Jan. 8, 2014, in the corresponding Japanese application No. 2012-530201.

Primary Examiner — Daniel E. Kolker
Assistant Examiner — James Rogers

(57) **ABSTRACT**

Herein is reported a tangential flow filtration method with a pre-filtration solute concentration adjustment in order to ensure a defined concentration of the components of the solution after tangential flow filtration.

13 Claims, 10 Drawing Sheets

Fig. 1

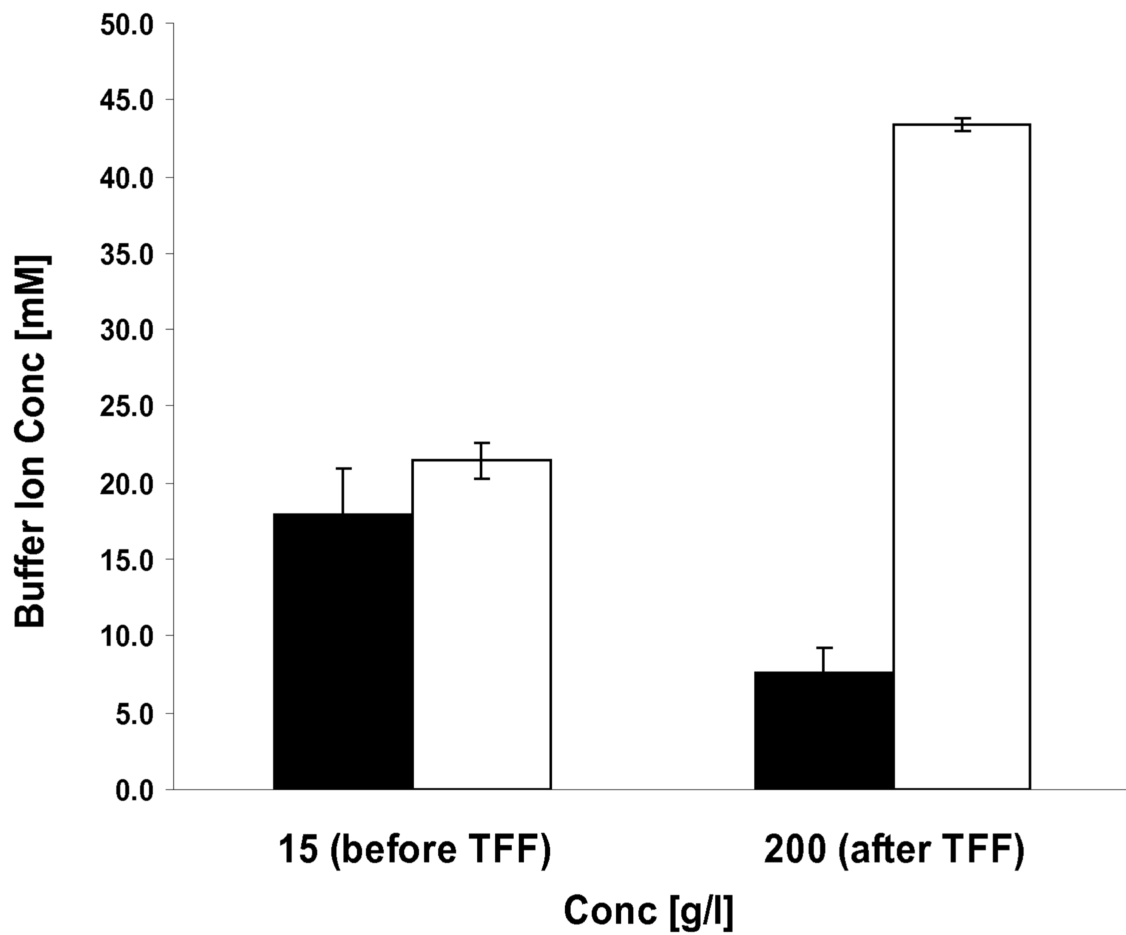


Fig. 2

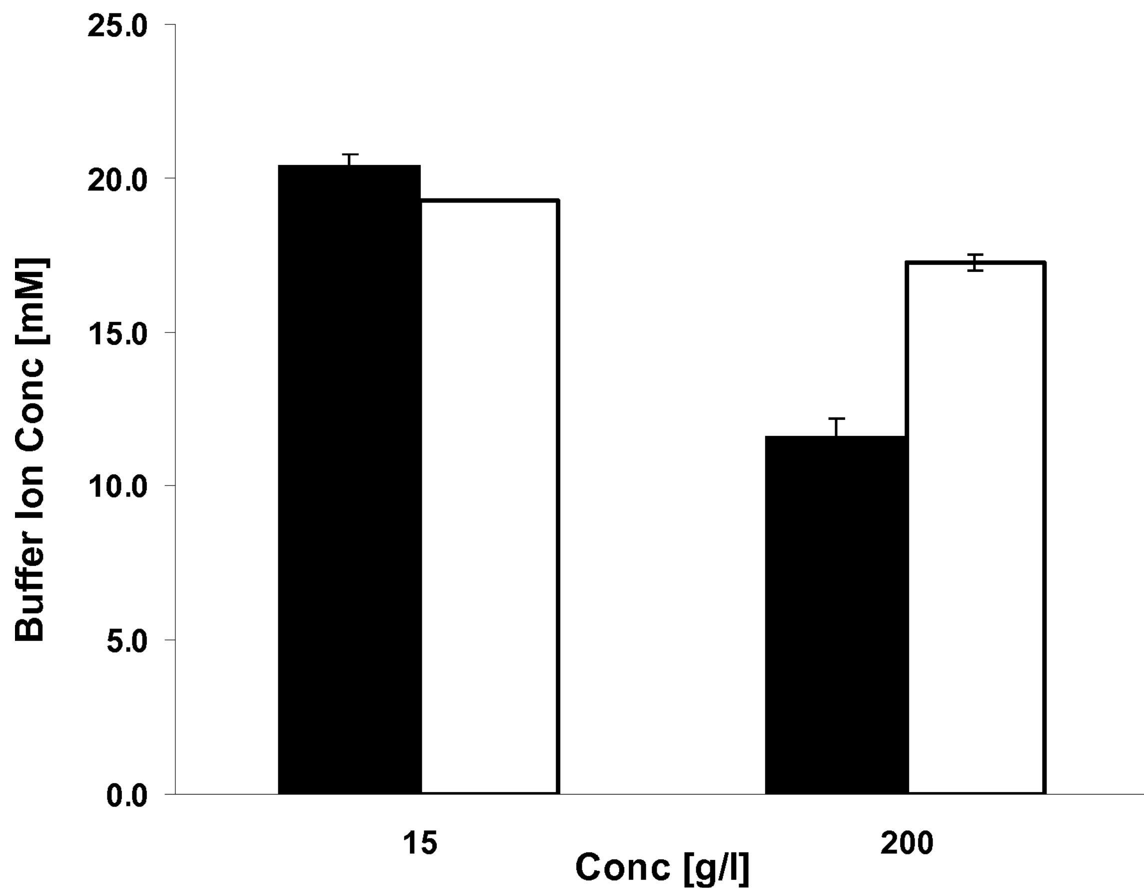


Fig. 3

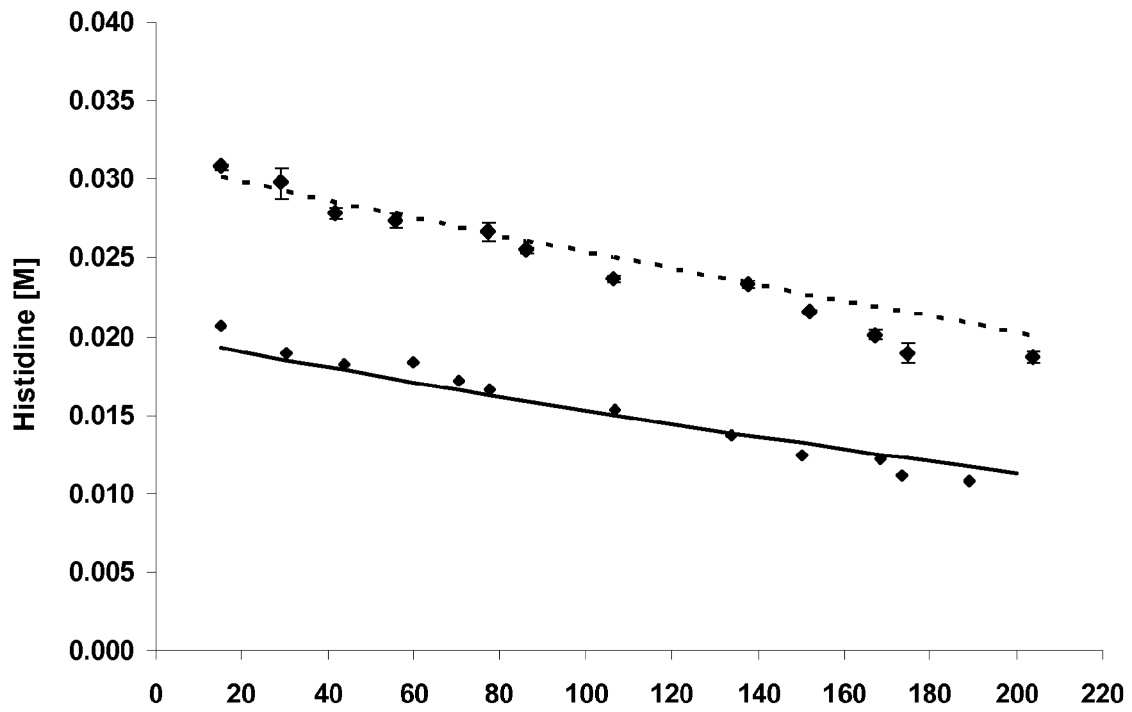


Fig. 4

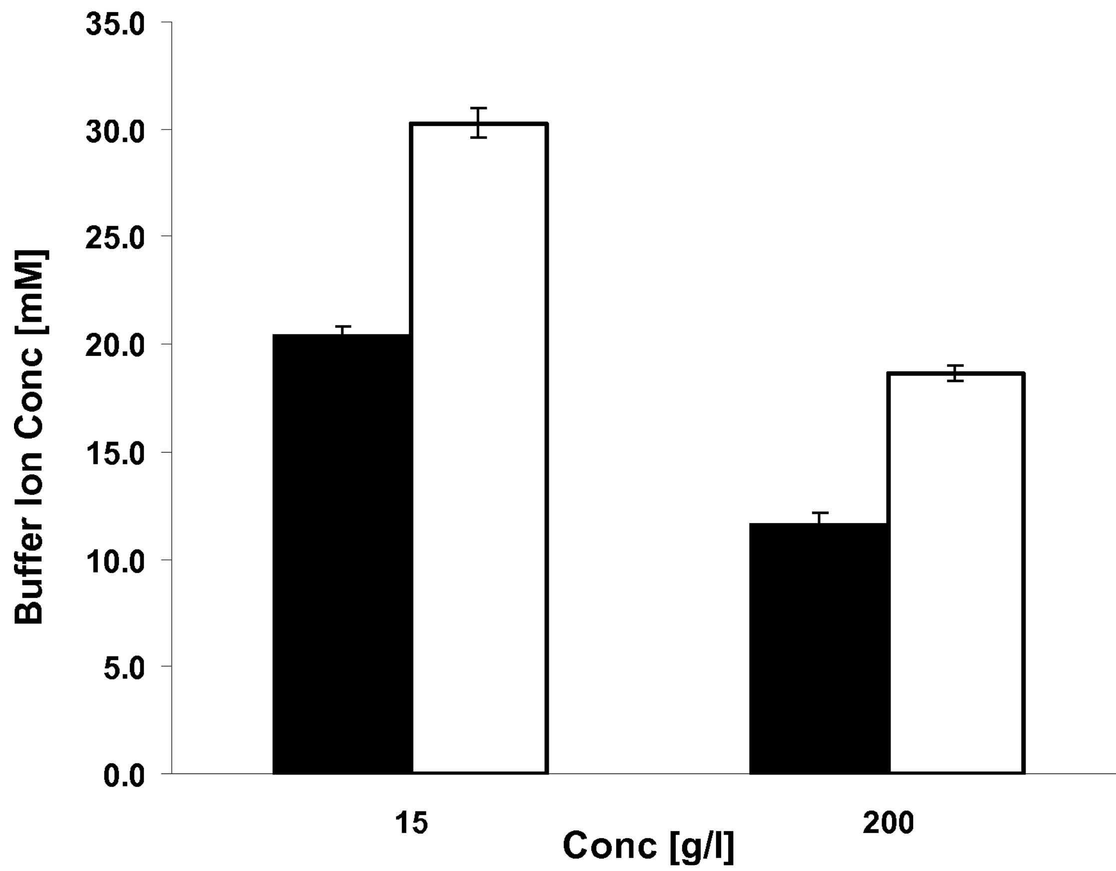


Fig. 5

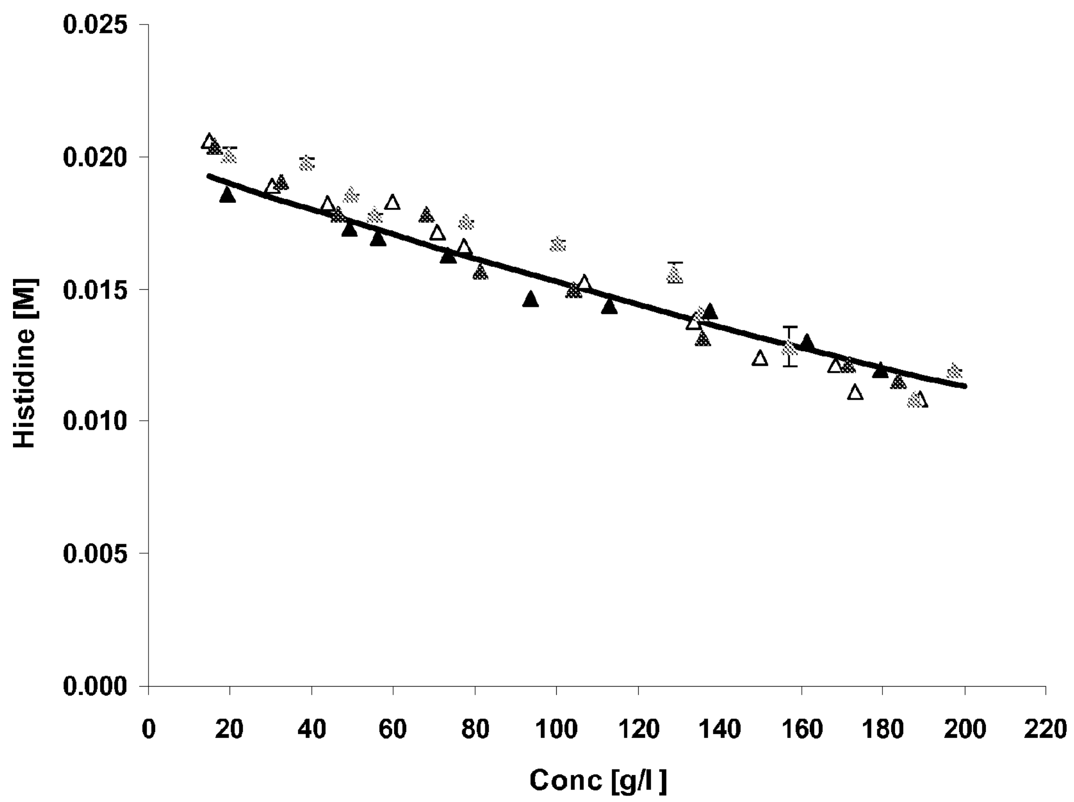


Fig. 6

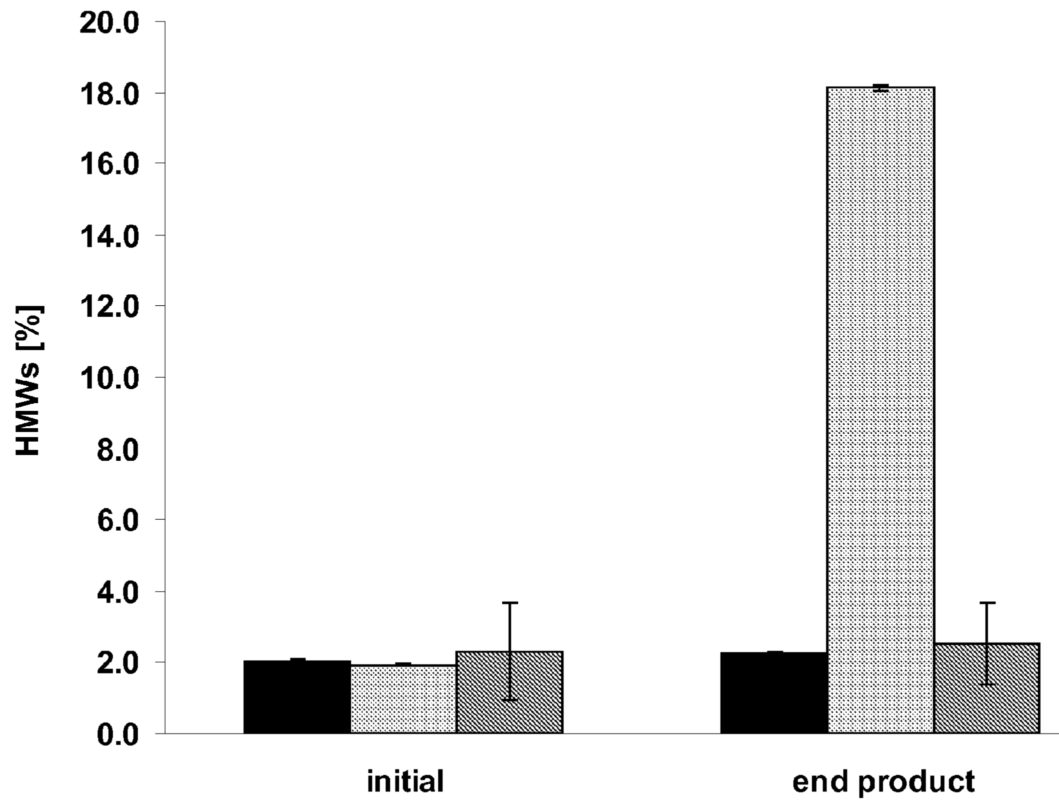


Fig. 7

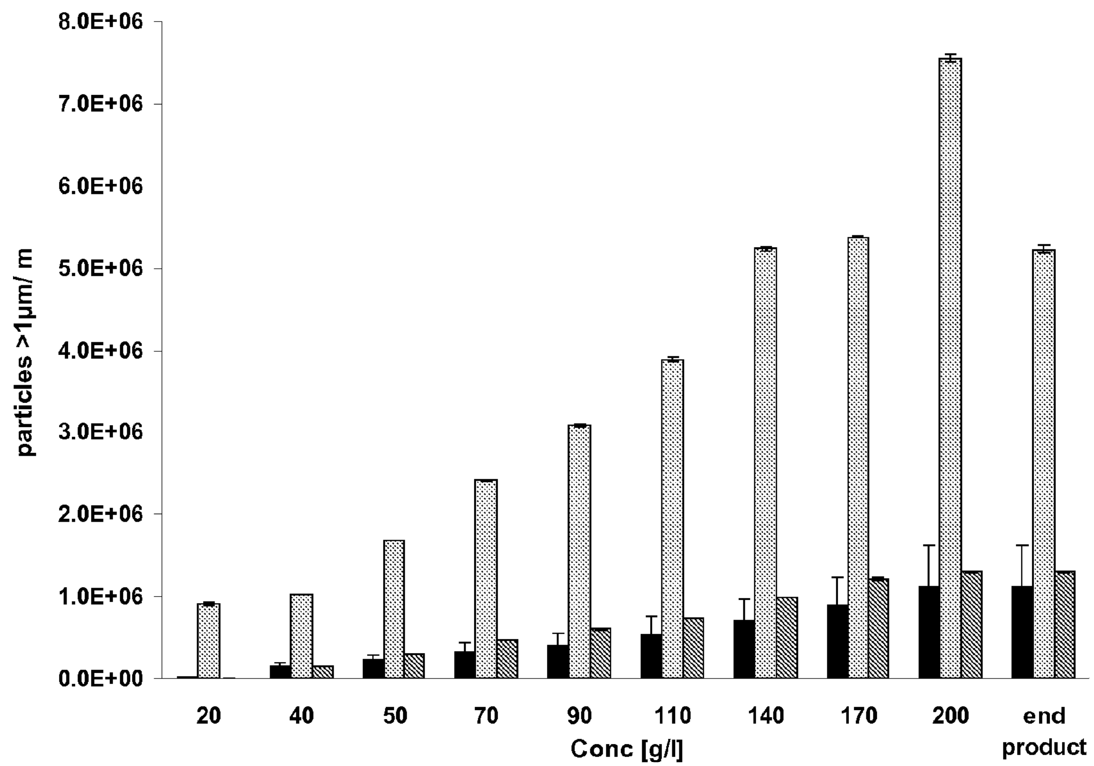


Fig. 8

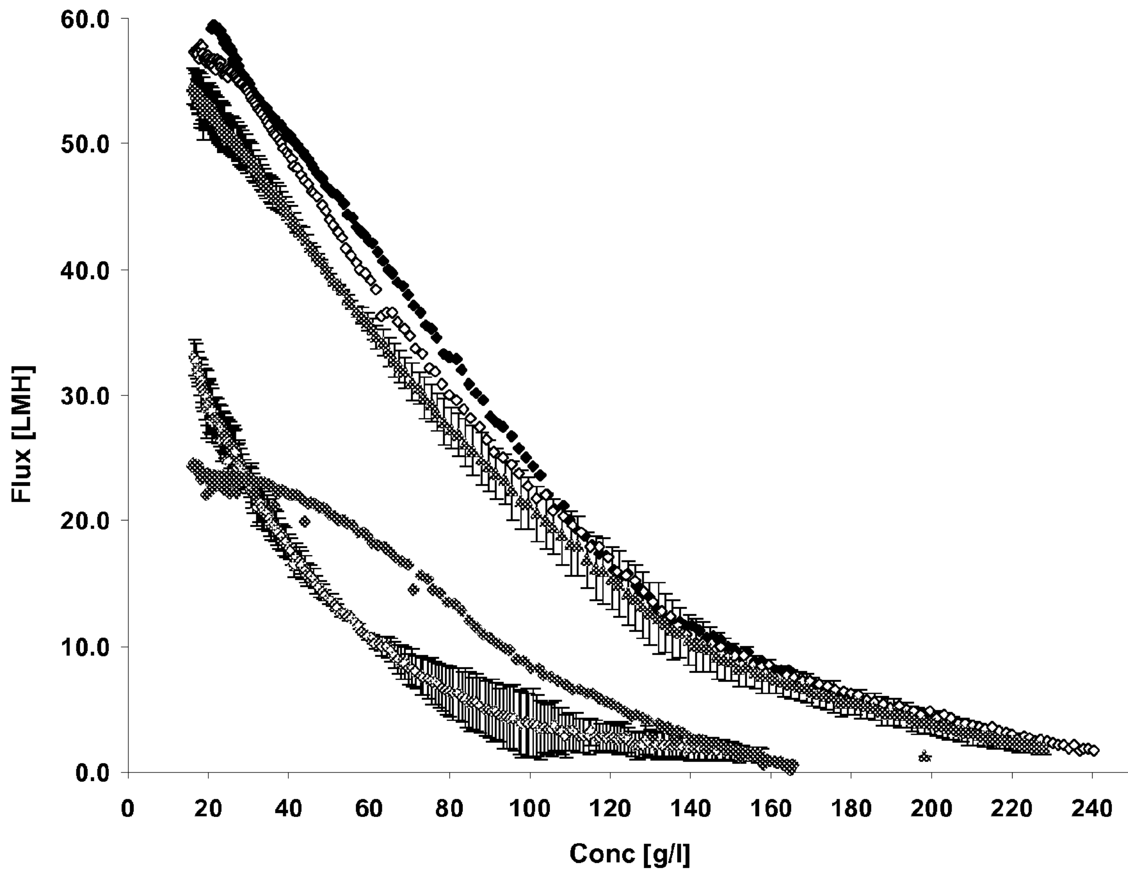


Fig. 9

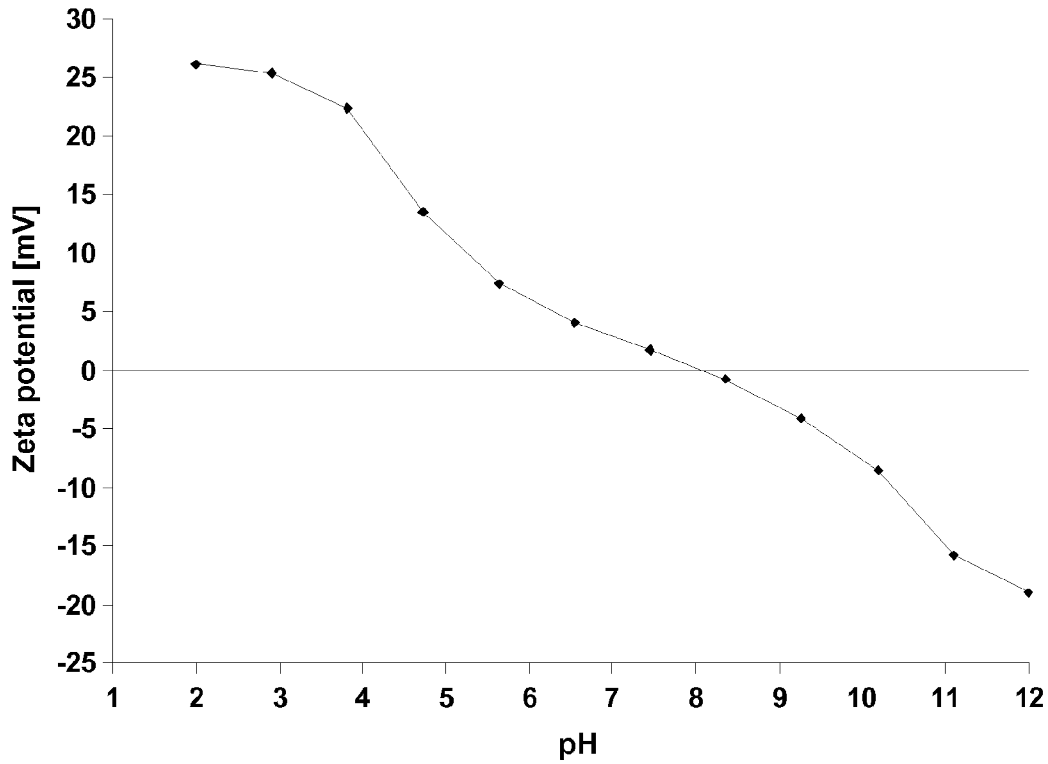
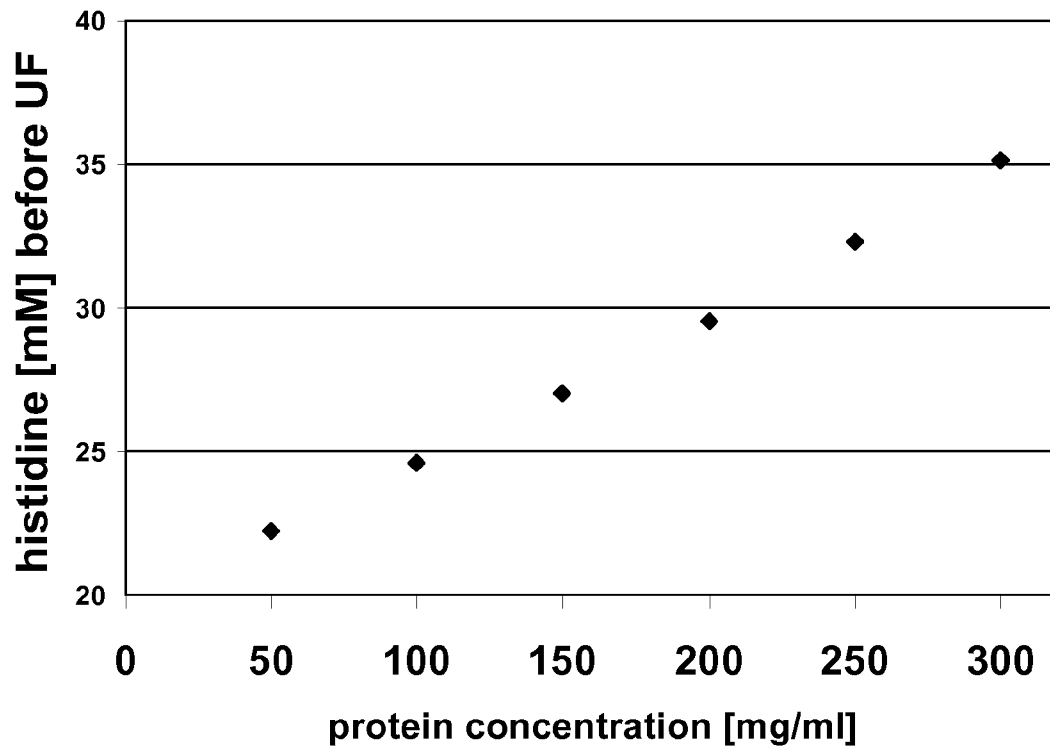


Fig. 10



PRE-FILTRATION ADJUSTMENT OF BUFFER SOLUTES

PRIORITY TO RELATED APPLICATION(S)

This application claims the benefit of European Patent Application No. 09012316.7 filed Sep. 29, 2009 and International Patent Application PCT/EP2010/062554, filed Aug. 27, 2010. The entire contents of the above-identified applications are hereby incorporated by reference.

Herein is reported a method for the pre-filtration adjustment of the solute concentration prior to a tangential flow filtration in order to obtain a bulk pharmaceutical active protein ingredient.

BACKGROUND OF THE INVENTION

Polypeptides play an important role in today's medical portfolio. Expression systems for the production of recombinant polypeptides are well-known. For human application every pharmaceutical substance has to meet distinct criteria. To ensure the safety of biopharmaceutical agents to humans, for example, nucleic acids, viruses, and host cell proteins, which would cause severe harm, have to be removed. To meet the regulatory specification one or more purification steps have to follow the manufacturing process. Among other, purity, throughput, and yield play an important role in determining an appropriate purification process.

Due to their chemical and physical properties, such as molecular weight and domain architecture, including secondary modifications, the downstream processing of immunoglobulins is essential. For example, concentrated solutions are required not only for formulated drugs but also for intermediates in downstream processing (DSP) to achieve low volumes for economic handling and application storage. Furthermore, fast concentration processes are favored to ensure smooth processes and short operating times. In this context tangential flow filtration (TFF) processes are used.

Saxena, A., et al. report membrane-based techniques for the separation and purification of proteins (Adv. Colloid Interfacial Sci. 145 (2009) 1-22. In WO 2009/010269 a variable tangential flow filtration method is reported. Mignard, D., et al. report fouling during the cross-flow ultrafiltration of proteins (J. Membr. Sci. 186 (2001) 133-143). An optimization diagram for membrane separations is reported by Van Reis, R., et al., J. Membr. Sci. 129 (1997) 19-29).

Thermodynamic non-ideality of protein containing solutions during membrane based processes has been reported by Donnan, F. G., Z. Elektrochem. 17 (1911) 572-581. Stoner et al. (J. Pharm. Sci. 93 (2004) 2332-2342) reported the concentration of charged solutes encompassing chloride, histidine and acetate during dialysis of the different proteins at various protein concentrations.

SUMMARY OF THE INVENTION

One aspect as reported herein is an ultrafiltration method for concentrating an immunoglobulin solution comprising the following steps:

a) providing an immunoglobulin solution with a pH value and with a first concentration S^+ or S^- of a buffer substance,

b) adjusting the first concentration of the buffer substance to a second concentration S' and maintaining the pH value, whereby the second concentration S' is calculated with equation 2 if the buffer substance is a cation/neutral pair or with equation 3 if the buffer substance is a neutral/anion pair,

c) concentrating the solution of b) by a tangential flow filtration,

wherein equation 2 is

$$S^+ = \frac{-zP + \sqrt{(zP)^2 + 4\left(\frac{S'}{\rho'}\left(\rho - \frac{PM_P}{1000}\right)\right)^2}}{2}$$

and equation 3 is

$$S^- = \frac{zP + \sqrt{(zP)^2 + 4\left(\frac{S'}{\rho'}\left(\rho - \frac{PM_P}{1000}\right)\right)^2}}{2}$$

with

the molar concentration in the retentate of positively/negatively charged solutes (S^+/S^-), the charge of the protein (z), the molar concentration (P) and the molecular weight (Mp) of the protein, the density of the solution in the retentate (ρ) and the permeate (ρ'), and the theoretical molar concentration of the diffusible solute (S'),

whereby the theoretical diffusible solute concentration S' is corrected by a correction factor that is considering the pH value wherein the ratio between buffer anion/buffer cation and buffer acid is calculated for each pH value determined in the retentate by using the Henderson-Hasselbach-Equation and the relative increase at each pH value is used as the respective correction factor.

In one embodiment the buffer substance is histidine and that the second concentration is calculated with equation 2. In one embodiment the pH value is of from pH 5.0 to pH 6.0. In a further embodiment the pH value is pH 5.5. In another embodiment the first concentration is approximately 20 mM. In a further embodiment the second concentration is of from 24 mM to 37 mM with a protein concentration of the concentrated solution of from 100 g/l to 300 g/l, respectively. In one embodiment the protein concentration is approximately 200 g/l and the second concentration is of from 28 mM to 31 mM. In another embodiment the first concentration is approximately 46 mM. In a further embodiment the second concentration is of from 52 mM to 72 mM with a protein concentration of the concentrated solution of from 100 to 300 g/l, respectively. In one embodiment the protein concentration is approximately 200 g/l and the second concentration is of from 59 mM to 62 mM.

In another embodiment the buffer substance is acetate and the concentration is calculated with equation 3. In one embodiment the pH value is of from pH 4.5 to pH 6.0. In a further embodiment the pH value is pH 5.5. In another embodiment the first concentration is approximately 20 mM. In a further embodiment the second concentration is of from 8 mM to 19 mM with a protein concentration of the concentrated solution of from 300 g/l to 100 g/l, respectively. In one embodiment the protein concentration is approximately 200 g/l and the second concentration is of from 12 mM to 17 mM. In another embodiment the first concentration is approximately 45 mM. In a further embodiment the second concentration is of from 41 mM to 48 mM with a protein concentration of the concentrated solution of from 300 to 100 g/l,

respectively. In one embodiment the protein concentration is approximately 200 g/l and the second concentration is of from 43 mM to 47 mM.

In one embodiment the immunoglobulin is an anti-P-selectin antibody or an anti-Aβ antibody.

Another aspect as reported herein is a method for producing an immunoglobulin in vitro comprising

a) cultivating a cell comprising a nucleic acid encoding the immunoglobulin,
b) recovering the immunoglobulin from the cultivation medium or the cell of step a)
c) purifying the immunoglobulin,
d) concentrating the immunoglobulin with a method as reported herein and thereby producing an immunoglobulin.

DETAILED DESCRIPTION OF THE INVENTION

Herein is reported a tangential flow filtration method with a pre-filtration solute concentration adjustment in order to ensure a defined concentration of the components of the solution after tangential flow filtration.

The term "cation/neutral pair" denotes a buffer substance which provides for a buffer system consisting of the buffer substance in neutral form and the buffer substance in protonated, i.e. positively charge form, as cation. One example thereof is histidine. The term "neutral/anion pair" denotes a buffer substance which provides for a buffer system consisting of the buffer substance in neutral form and the buffer substance in de-protonated, i.e. negatively charge form, as anion. One example is acetate.

The term "tangential flow filtration", or short "TFF", denotes a filtration process wherein a solution containing a polypeptide to be concentrated flows along, i.e. tangential, to the surface of a filtration membrane. The filtration membrane has a pore size with a certain cut off value. In one embodiment the cut off value is in the range of 20 kDa to 50 kDa, in another embodiment of 30 kDa. TFF was conducted as ultra filtration. The term "cross-flow" denotes the flow of the solution to be concentrated tangential to the membrane (retentate flow). The term "flux" or "permeate flow", which can be used interchangeably, denotes the flow of fluid through the membrane, i.e. through the pores of the membrane. That is, it denotes the volumetric rate of the permeate flow through the membrane. A flow is usually given in terms of volume per unit membrane area per unit time as liters/m^2/h (LMH). The permeate comprises the solvent of the solution to be concentrated on the retentate side as well as molecules with a molecular weight below the cut off value of the employed membrane but not the polypeptide to be concentrated. The terms "transmembrane pressure" or "TMP", which can be used interchangeably, denote the pressure which is applied to drive the solvent and components smaller than the cut off value of the membrane through the pores of the membrane. The transmembrane pressure is an average pressure of the inlet, outlet and permeate and can be calculated as:

$$TMP = \frac{(p_{in} + p_{out})}{2} - p_{permeate}. \quad \text{(equation 1)}$$

The term "solute" as used herein denotes all components, i.e. ionic and non-ionic, of a solution to be concentrated except the water molecules and the molecules of the polypeptide to be concentrated. Generally comprises the solution to be concentrated a polypeptide, water and a buffer salt, and optionally a non-buffer salt, such as sodium chloride.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 20 amino acid residues are referred to as "peptides". A "protein" is a macromolecule comprising one or more polypeptide chains or at least one polypeptide chain of more than 100 amino acid residues. A polypeptide may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a polypeptide by the cell in which the polypeptide is produced, and will vary with the type of cell. Polypeptides are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

The term "immunoglobulin" refers to a protein consisting of one or more polypeptide(s) substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the different constant region genes as well as the myriad immunoglobulin variable region genes. Immunoglobulins may exist in a variety of formats, including, for example, Fv, Fab, and $F(ab)_2$ as well as single chains (scFv) or diabodies (in general, Hood, L. E., et al., Immunology, The Benjamin N.Y., 2nd edition (1984)). The term "immunoglobulin", thus, denotes a complete immunoglobulin consisting of two immunoglobulin heavy chains and two immunoglobulin light chains, as well as an "immunoglobulin fragment" comprising at least one domain selected from the variable domain, the C_H1 domain, the hinge-region, the C_H2 domain, the C_H3 domain, or the C_H4 domain of a heavy chain, or the variable domain or the C_L domain of a light chain and an "immunoglobulin conjugate" comprising at least one domain of an immunoglobulin heavy or light chain conjugated via a peptide bond to a further polypeptide. The further polypeptide is a non-immunoglobulin peptide, such as a hormone, or toxin, or growth receptor, or antifusogenic peptide, or complement factor, or the like.

For the purification of biotechnologically produced immunoglobulins often a combination of different column chromatography steps is employed. In one embodiment a protein A affinity chromatography is followed by one or two additional separation steps. The final purification step is a so called "polishing step" for the removal of trace impurities and contaminants like aggregated immunoglobulins, residual HCP (host cell protein), DNA (host cell nucleic acid), viruses, or endotoxins. For this polishing step in one embodiment an anion exchange material in a flow-through mode is used.

Different methods are well established and widespread used for protein recovery and purification, such as affinity chromatography with microbial proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and electrophoretical methods (such as gel electrophoresis, capillary electrophoresis). These methods can be combined independently in different embodiments of the current invention.

The term "immunoglobulin in monomeric form" and grammatical equivalents thereof denotes an immunoglobulin molecule not associated with a second immunoglobulin molecule, i.e. neither covalently nor non-covalently bound to

another immunoglobulin molecule. The term "immunoglobulin in aggregated form" and grammatical equivalents thereof denotes an immunoglobulin molecule which is associated, either covalently or non-covalently, with at least one additional immunoglobulin molecule or fragment thereof, and which is eluted in a single peak from a size exclusion chromatography column. The term "in monomeric form" and grammatical equivalents thereof as used within this application not necessarily denotes that 100% of an immunoglobulin molecule are present in monomeric form. It denotes that an immunoglobulin is essentially in monomeric form, i.e. at least 90% of the immunoglobulin is in monomeric from, in one embodiment at least 95% of the immunoglobulin is in monomeric form, in another embodiment at least 98% of the immunoglobulin is in monomeric form, in a further embodiment at least 99% of the immunoglobulin is in monomeric form, and in a final embodiment more than 99% of the immunoglobulin is in monomeric form determined as peak area of a size exclusion chromatogram of the immunoglobulin preparation. The term "in monomeric and in aggregated form" denotes a mixture of immunoglobulin molecules not associated with other immunoglobulin molecules and of immunoglobulin molecules associated with other immunoglobulin molecules. In this mixture neither of the monomeric form nor the aggregated form is present exclusively. The term "high molecular weight (HMW) form" denotes polymeric, i.e. aggregated, immunoglobulin, whereby the aggregate is still soluble in an aqueous buffered solution.

The term "100%" as used within this application denotes that the amount of components other than a specified component are below the detection limit of the referred to analytical method under the specified conditions.

The terms "90%", "95%", "98%", "99%" as used within this application denote no exact values but values within the accuracy of the referred to analytical method under the specified conditions.

Generally ion exchange chromatography in flow-through mode is the final chromatographic step in purification processes of monoclonal immunoglobulins to remove residual host cell DNA, endotoxins and retrovirus-like particles. Hence, the purified ion exchange chromatography pool is in e.g. a phosphate- or tris (hydroxymethyl)-aminomethan buffer. Subsequently, the conditions have to be changed to a buffer system for e.g. ensuring stability of the active pharmaceutical ingredient during storage. Generally the pH value is slightly acidic e.g. between pH 5 and pH 6 and a conductivity of less than 5 mS/cm is required (Daugherty, A. L. and Mrsny, R. J., Adv. Drug Deliv. Rev. 58 (2006) 686-706).

Concomitantly the ion exchange pool is the basis buffer for formulation by using/adding different stock solutions of excipients like surfactants and sugars. Therefore, the ion exchange chromatography pool is concentrated and diafiltered into a suitable buffer composition by ultrafiltration to provide a defined composition of protein, buffer solutes, pH and conductivity.

Electrostatic interactions of ions and polypeptides at non-isoelectric pH values lead to unequal partitioning thereof during an ultrafiltration process on the retentate and permeate side of the ultrafiltration membrane. This results in a significant variation of solute concentration before and after tangential flow filtration (for concentration and diafiltration) and results in variations in pH and conductivity before, during and after the tangential flow filtration.

For example, an immunoglobulin ion exchange chromatography pool was diafiltered against 20 mM histidine buffer (pH 5.5; 1.6 mS/cm) of a 1 to 10-fold diafiltration volume (DV) with respect to the pool volume. Thereafter, the diafiltered pool was concentrated to more than 210 mg/ml protein concentration in a tangential flow filtration. It has been found that even after applying a 10-fold diafiltration volume the predefined conditions for the histidine buffer system concerning pH value and conductivity could not be maintained constant after the complete concentration process. After starting the concentration process the pH value increases to pH 5.7 and conductivity reaches 2.2 mS/cm at a protein concentration of 215 mg/ml in the retentate.

Moreover, conductivity and pH value were monitored during the UF concentration process in 20 mM histidine buffer at pH 5.5. With increased protein concentration an increase in conductivity was observed again. In addition, an increase in the pH value up to pH 5.8 was observed as well.

A different but also similar observation was made during an UF concentration processes with another buffer system (20 mM acetate buffer at pH 5.5). During the UF process the pH value increased to pH 5.8 similar as observed with the 20 mM histidine buffer, but the conductivity decreased with increasing protein concentration. Essentially the same was observed at a higher acetate concentration of 45 mM at pH 5.0.

During the UF concentration process of the monoclonal immunoglobulin in two different, defined buffer systems, a significant accumulation of the buffer substance in the case of acetate in the retentate, and a significant loss of the buffer substance in the case of histidine in the retentate has been observed (see e.g. FIGS. **1** and **3**). The concentration of acetate nearly doubled whereas the concentration of histidine was halved at an immunoglobulin concentration of about 200 mg/ml. Both induced changes in conductivity and pH during the concentration process.

The unequal partitioning of the solute compounds during diafiltration and concentration operations results in excipient concentrations, pH and conductivity values which are significantly different from those of the diafiltration buffer at the start of the process. As this can influence stability of the final formulated immunoglobulin the preset solute compound concentration are required to be present in the concentrated immunoglobulin preparation.

Different possibilities to correct the changes during tangential flow filtration are likely:

- restock/dilute with buffer solution after concentration,
- adjustment of the pH value close to the isoelectric point value before the tangential flow filtration (see e.g. FIG. **2**),
- defined addition/reduction of solute prior to tangential flow filtration (see e.g. FIG. **4**).

The dilution after the UF with buffer solution is not suited as this will result also in a dilution and reduction of the immunoglobulin concentration. This is in direct opposite to the intention of the UF process to provide concentrated immunoglobulin solutions.

It has now been found that the defined addition/reduction of solute concentration prior to the tangential flow filtration process is advantageous in order to correct the concentration changes independent of the concentration device, membrane material, and concentration parameters. It has been found that as in one embodiment in case of a histidine buffer (=solute) an adjustment at about pH 5.0 to 29.6 mM and 60 mM histidine, respectively, before tangential flow filtration is required to achieve a predefined histidine buffer concentration of 20 mM and 46 mM histidine, respectively, after the tangential flow filtration in the concentration of an immunoglobulin of the IgG 1 and IgG 4 class to 215 mg/ml.

In an alternative way of addressing the concentration changes during the ultrafiltration process the concentrates were readjusted to a pH value of pH 5.0 by adding 0.5 M

hydrochloric acid (HCl) to the stirred solution after the UF process. This was only necessary for the solutions which were concentrated at pH 7.5 and at 20 mM histidine pH 5.0 due to the pH shift during UF. The experiments conducted at 29.6 mM histidine pH 5.0 did not show a pH shift as reported before. Table 1 shows the pH values before UF, after UF and after readjustment to pH 5.0.

TABLE 1

starting conditions	pH before UF	pH after UF	pH end product
20 mM histidine buffer, pH 5.0	4.97 +/− 0.04	5.40 +/− 0.06	4.90 +/− 0.14
32 mM histidine buffer, pH 5.0	5.0 +/− 0.04	5.20 +/− 0.05	—
20 mM histidine buffer, pH 7.4	7.37 +/− 0.06	7.47 +/− 0.05	4.81 +/− 0.22

pH values before UF, after UF and for the end product with readjusted pH to 5.0;
0.5M hydrochloric acid was taken to readjust the pH to pH 5.0 in the end product;
mean values of three measurements are presented ± SD.

The adjustment of the solute concentration prior to the UF is calculated based on the following equations 2 and 3.

$$S^{+}=\frac{-zP+\sqrt{(zP)^2+4\left(\frac{S'}{\rho'}\left(\rho-\frac{PM_P}{1000}\right)\right)^2}}{2} \quad \text{(equation 2)}$$

Equation 2 describes the molar concentration in the retentate of positively charged solutes (S^+) being able to pass the membrane. S^+ depends on the charge of the protein (z), the molar concentration (P) and the molecular weight (Mp) of the protein, as well as on the density of the solution in the retentate (ρ) and the permeate (ρ'). S' is the theoretical molar concentration of the diffusible solute.

$$S^{-}=\frac{zP+\sqrt{(zP)^2+4\left(\frac{S'}{\rho'}\left(\rho-\frac{PM_P}{1000}\right)\right)^2}}{2} \quad \text{(equation 3)}$$

Equation 3 describes the molar concentration in the retentate of negatively charged solutes (S^-) being able to pass the membrane.

For an exemplary immunoglobulin against the amyloid β peptide (anti-Aβ antibody) as reported in WO 2003/070760 or US 2005/0169925 the calculation is made as outlined in the following:

For obtaining a solution with a final immunoglobulin concentration of 200 mg/ml in 20 mM histidine buffer at pH 5.5. The histidine concentration before the concentration step is calculated with equation 2 rearranged to calculate S' as the solute molecule is a positively charged molecule.

The rearranged equation 2 (equation 2') is:

$$S'=\frac{\sqrt{\frac{(2*[S^{+}]+z*P)^2-(z*P)^2}{4}}}{\rho-\frac{P*M_p}{1000}}*\rho' \quad \text{(equation 2')}$$

The following parameter values were employed:
molecular mass of the immunoglobulin: 150,000 g/mol
density of the permeate: 0.9989 g/ml
molar concentration at end concentration: 0.00133 mol/l
charge of the protein: +9
(determination see Example 13)
starting protein concentration: 15 mg/ml
(determination see Example 4)
target buffer concentration: 0.020 M
density of the protein solution at end of concentration: 1.0551 g/ml
(determination see Example 14)
The values have been entered into equation 2' to result in:

$$[\text{histidine concentration prior to the concentration}] = \frac{(((2*0.020+9*0.00133)^2-(9*0.00133)^2)*0.25)^{1/2}}{1.0551-(0.00133*150{,}000)/1000}*0.9989\ \text{mol/l} = 0.02955\ \text{mol/l} = 29.6\ \text{mM}$$

Therefore, in order to make the calculation e.g. for a final concentration of 200 mg/ml, the charge of the immunoglobulin to be concentrated according to example 13, the density of the immunoglobulin solution after the concentration according to example 14, and the concentration of the immunoglobulin in the starting immunoglobulin solution according to example 4 have to be determined experimentally.

It is also possible to use literature values for the density of the starting solution according to the following Table 2.

TABLE 2

Density of protein solutions.

protein concentration [mg/ml]	density of the solution [g/ml]
15	1.0105
28	1.0140
41	1.0174
55	1.0211
68	1.0246
85	1.0291
100	1.0331
112	1.0362
120	1.0384
138	1.0431
185	1.0556
200	1.0596

The rearranged equation 3 (equation 3') is:

$$S'=\frac{\sqrt{\frac{(2*[S^{+}]-z*P)^2-(z*P)^2}{4}}}{\rho-\frac{P*M_p}{1000}}*\rho' \quad \text{(equation 3')}$$

In case of an anionic buffer salt (solute) due to the increase of the pH value during the UF process the percentage of buffer salt (solute) in anionic form also increases. Therefore more buffer salt anions will actually be lost than restocked based on the calculation by using the above equations without considering the pH value. Therefore, the theoretical diffusible solute concentration S' has to be corrected by using a factor considering the pH value. The ratio between buffer anion/buffer cation and buffer acid can be calculated for each pH value determined in the retentate by using the Henderson-Hasselbach-Equation. The relative increase at each pH value can be used as the respective correction factor.

The molar solute concentration was approximated by inserting the actual charge value of the immunoglobulin at the pH value in equations 2 and 3, respectively. The overall protein charge was determined by zeta potential measurements (see example 13).

Several possibilities to determine the protein valence depending on the pH value are available. Beside the calculated titration curves based on protein sequence by combining the average pK_a-values of all acidic and basic amino acid side chains, the experimental determination based on electrophoretic mobility, like the zeta potential measurement (Faude, A., et al., J. Chromatogr. A 1161 (2007) 29-35; Salinas, B. A., J. Pharm. Sci. 99 (2009) 82-93) or gel- and capillary electrophoresis (Winzor, D. J., et al., Anal. Biochem. 333 (2004) 225-229) is possible. Since the protein valence depends not only upon pH but also on the buffer electrolyte composition of the environment, there is no realistic alternative available to determine the actual charge of the protein.

Two immunoglobulin solutions (see example section for details) were concentrated from 15 mg/ml to 200 mg/ml. One solution used contained 20 mM histidine at pH 5.0 and one solution used contained 20 mM histidine at pH 5.5 before UF. Both immunoglobulin solutions were intended to have 20 mM histidine present at the predefined pH after the concentration process. During UF the displacement of histidine was observed, which was quantitatively corrected by employing equation 2 with a protein charge of +11. At 200 mg/ml immunoglobulin concentration only 10.8 mM histidine remained without restock prior to the UF. It was calculated that 29.6 mM histidine should be present before the UF, to end up with 20 mM histidine after processing at 200 mg/ml protein concentration. Experimental data conducted in a buffer system containing 30.3±0.7 mM histidine at pH 5.5 showed that concentrates at 200 mg/ml exhibit a histidine concentration of 18.6±0.4 mM as predicted. Thus, it was confirmed that by using a higher histidine concentration at the beginning of the UF, calculated by using equation 2', the intended histidine concentration was present after UF up to a target protein concentration of 200 mg/ml.

Moreover, the intended histidine concentration of 46 mM at a protein concentration of 200 mg/ml can be achieved by applying an initially higher histidine concentration of 60 mM before UF as calculated by using equation 2'. In this case a protein charge of +7 was applied.

In case of histidine, the calculated higher solute molarity before starting the UF concentration process resulted in the intended molarities of histidine in the concentrated bulks. Moreover, during and after the UF process the pH value remained almost constant compared to experiments conducted at a lower molarity.

At a protein concentration of 200 mg/ml the pH value was shifted from pH 5.44±0.04 to pH 5.80±0.05, if the histidine concentration was not increased to 29.6 mM histidine before the UF. If the histidine concentration was increased to 29.6 mM, the pH was almost constant, i.e. pH 5.45±0.04 before and pH 5.57 after UF processing.

It has further been found that an adjustment of the pH value is not necessary.

If the pH value is adjusted close to the isoelectric point prior to the tangential flow filtration process the formation of aggregates and particles is induced during the tangential flow filtration process. In contrast thereto the systematic correction of concentration parameters prior to the tangential flow filtration process does not induce the formation of aggregates and/or particles.

Immunoglobulin solutions at a concentration of 20 mg/ml were ultrafiltrated up to 200 mg/ml. Buffer systems based on histidine were used. On the one hand, experiments were conducted in a 29.6 mM histidine buffer pH 5.0. On the other hand UF was performed in a 20 mM histidine buffer pH 7.4. Results were compared to experiments conducted in a buffer system containing 20 mM histidine at pH 5.0 (see FIGS. **6** and **7**).

It was observed, that particle formation during of UF was enhanced at pH 7.4. In the course of UF up to $8*10^6$ particles larger than 1 μm were formed and the turbidity increased from 0.1 AU to 1.6 AU. Solutions containing different immunoglobulins ultrafiltrated at a pH value of pH 5.0 were analyzed with respect to particle formation and turbidity. The formation of particles and the turbidity was clearly reduced at this pH value. This can be attributed to the pH value of pH 7.4 which is close to the isoelectric point (IP) of one of the antibodies, which was determined to be about 8 (Nakatsuka, S. and Michaels, A. S., J. Membr. Sci. 69 (1992) 189-211).

Particle measurement, turbidity at 350 nm and SE-HPLC were performed to monitor the induction of aggregate due to the addition of hydrochloric acid. For the readjusted end product an induction of particles larger 1 μm and an increase in turbidity was observed for the solutions concentrated in 20 mM histidine at pH 5.0.

An increase of HMWs from 2.23±0.05% to 2.71% was determined. For the solution containing 29.6 mM histidine at pH 5.0 prior to the UF, the number of particles per ml larger than 1 μm, the turbidity values and the percentage of HMWs remained constant.

After readjusting the pH value from pH 7.4 to pH 5.0 the percentage in HMWs increased from 0.74% to 17.15±0.97%. Concomitantly, the number of particles larger than 1 μm and the turbidity values decreased. Nevertheless, the number of particles and the turbidity values remained much higher compared to the two other processes.

It has been observed, that the addition of a higher molar HCl increases the percentage of dimers as well as of oligomers. This was prevented, by adding a 0.02 M HCl. Concomitantly, the addition of a diluted HCl resulted in a massive dilution of the concentrated protein bulk, ending up with about a third in protein concentration after pH adjustment.

It has also been found that with a change of the pH value prior to the tangential flow filtration process a reduction of the transmembrane flux is associated resulting in a dramatically increased concentration time (see FIG. **8**). If a correction of the solute concentration is performed prior to the UF no reduction of the transmembrane flux occurs and the concentration time is unaffected.

The permeate flux was observed to be significantly reduced during processing compared to the experiments conducted at pH 5.0. The process time was more than doubled from 120±2 min. to 300±2 min. when a pH value of pH 7.4 was adjusted before the UF was started. It has been observed, that the addition of histidine prior to the UF step had no influence on the permeate flux.

It has also been found that with the altered buffer composition during the tangential flow filtration process the stability of the concentrated protein is affected.

UF processes are conducted during process and formulation development. Different UF systems, membrane material and operational parameters are applied to concentrate the protein bulk. In order to proof, that the herein presented model is adequately reflecting the experimental values concerning the loss of histidine during UF concentration, different membrane materials, UF systems and operational parameters were tested (see Table 3 and FIG. **5**).

TABLE 3

Different set-ups.				
histidine concentration [mM]	pH value	UF membrane material/ pore size [kDa]	UF system	Δp [bar]
20	5.0	RC/30	stirred cell	2.0
20	5.0	RC/30	cross flow	0.8
20	5.0	RC/30	cross flow	1.5
20	5.0	PES/30	cross flow	1.5

Especially polyethersulfone (PES) is known to adsorb protein to a higher extent than regenerated cellulose (RC), due to enhanced hydrophobicity. Moreover, proteins and solutes can interact with the membrane surface based on charge-charge interactions. As a consequence, the outcome of the solute molarity in the concentrates can be influenced by the choice of the membrane material.

Independent from the applied UF system, membrane material or operational conditions, the experimental data concerning the histidine molarity during UF can be approximated by using the equations as reported before.

In FIG. **10** a graph showing the concentration of histidine to be adjusted prior to the ultrafiltration depending on the intended final protein concentration in order to have a final solution with 20 mM histidine buffer at pH 5.5 exemplified with an anti-Aβ antibody calculated according to the method as reported herein is shown.

The following examples and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

DESCRIPTION OF THE FIGURES

FIG. **1** Buffer ion concentration before and after tangential flow filtration exemplified with a histidine and an acetate buffer exemplified with an anti-Aβ antibody; black=histidine buffer; white=acetate buffer.

FIG. **2** Buffer ion concentrations before and after tangential flow filtration exemplified with a histidine and an acetate buffer with adjustment of the pH value close to the isoelectric point prior to the tangential flow filtration; black=histidine buffer 20 mM pH 5; white=histidine buffer 20 mM pH 7.5.

FIG. **3** Course of the buffer ion concentration during tangential flow filtration exemplified with a histidine and an acetate buffer with adjustment of the buffer ion concentration prior to the tangential flow filtration; small diamonds: experimental data of 20 mM histidine buffer, solid line: fit for this data; large diamonds: experimental data of 29.6 mM histidine buffer, dashed lined: fit of this data.

FIG. **4** Buffer ion concentrations before and after tangential flow filtration exemplified with a histidine and an acetate buffer with adjustment of the buffer ion concentration prior to the tangential flow filtration; black=histidine buffer 20 mM; white=histidine buffer 29.6 mM.

FIG. **5** Change of the buffer ion concentration in a concentration process with different concentration device; solid line: fit; white triangles: Hydrosart™; grey triangles: stirring cell; black small triangles: PESU; black large triangles: ΔP=0.8 bar.

FIG. **6** Induction of high molecular weight compounds during concentration due to pH adjustment; black=concentrated at pH 5.0; light grey=concentrated at pH 7.5; dark grey=concentrated at pH 5 and addition of additional histidine prior to concentration.

FIG. **7** Induction of particles during concentration due to pH adjustment; black=concentrated at pH 5.0; light grey=concentrated at pH 7.5; dark grey=concentrated at pH 5 and addition of additional histidine prior to concentration.

FIG. **8** Dependency of the transmembrane flux on the retentate concentration and on the adjustment method; white small diamond=pH 7.5; grey diamond=pH 5.0 with a P of 0.8 bar; black diamond=pH 5.0 PESU; white large diamond=pH 5.0 Hydrosart™; black triangle=pH 5.0 with addition of histidine prior to concentration.

FIG. **9** Zeta potential determined according to Example 13 for an anti-IL-1R antibody.

FIG. **10** Graph showing the concentration of histidine to be adjusted prior to the ultrafiltration depending on the intended final protein concentration in order to have a final solution with 20 mM histidine buffer at pH 5.5 exemplified with an anti-Aβ antibody.

EXAMPLE 1

Material and Methods

Chemicals

All chemicals and reagents used were at least analytical grade. Hydrochloric acid and sodium hydroxide were taken from Merck KG (Darmstadt, Germany). L-histidine from Ajinomoto (Raleigh, USA) was used. Acidic acid was taken from Fluka (Steinheim, Germany). Sodium chloride was taken from Merck KG (Darmstadt, Germany).

Antibody

The method as reported herein is exemplified with an immunoglobulin against the amyloid β peptide (anti-Aβ antibody) as reported in WO 2003/070760 or US 2005/0169925. Another exemplary immunoglobulin is an anti-P-Selectin antibody as reported in WO 2005/100402 or US 2005/0226876.

EXAMPLE 2

Sample Preparation

A solution of the immunoglobulin in 20 mM histidine buffer at pH 5.5 and at a concentration of 50 mg/ml was taken for the concentration experiments in histidine buffer. To obtain material containing a higher molarity of histidine, histidine base was added to the protein solution and the pH was adjusted to pH 5.5 by adding 0.1 M hydrochloric acid.

To obtain solutions containing 20 mM and 45 mM acetate buffer pH 5.0, respectively, the 20 mM histidine material was diafiltered against the 10-fold volume of sodium acetate buffer pH 5.0 by using TFF.

A solution of an immunoglobulin in 20 mM histidine buffer at pH 5.0 and a concentration of 20 mg/ml was taken for the experiments in histidine buffer.

Before ultrafiltration (UF) processing the solutions were diluted to 10 mg/ml protein concentration by using the corresponding buffer and filtered through a 0.22 μm membrane cartridge (Sartorius, Göttingen, Germany).

EXAMPLE 3

Tangential Flow Filtration

For the preparation of concentrated immunoglobulin solutions the automated tangential flow filtration (TFF) system

ÄKTAcrossflow™ (GE Healthcare, Uppsala, Sweden) was used. The method as reported in WO 2009/010269 was used for all experiments.

In short a retentate flow rate of 240 $l/m^2/h$ and a TMP of 1.25 bars were chosen as the condition at the beginning of the UF process for an immunoglobulin concentration of from 5 mg/ml to 25 mg/ml. From 25 mg/ml to 50 mg/ml the TMP was lowered to 0.85 bars. In addition, the retentate flow rate was increased to 450 $l/m^2/h$, bringing the permeate flux from 30 up to 45 $l/m^2/h$. For a concentration range of from 50 mg/ml up to target concentration of 140 mg/ml or more a TMP of 0.85 bars and an increased retentate flow rate of 390 $l/m^2/h$ were set.

A Sartocon Slice flat sheet cassette with a Hydrosart™ membrane of regenerated cellulose, with a nominal molecular weight cutoff (NMWC) of 30 kDa and a membrane area of 0.02 m^2 was used (Sartorius, Göttingen, Germany). The total membrane loading was about 400 g/m^2 for each experiment. After the concentration the membrane module was cleaned with 1 M sodium hydroxide. The normalized flux rate for water (NWF) was determined after every cleaning cycle and compared to the obtained value before initial use. The cassette was only applied for the next experiments, if the NWF decline in $(l/m^2/h)/1$ bar at 20° C. was below 10% of the initial value to ensure complete cleaning and comparable membrane properties.

EXAMPLE 4

Concentration Determination

Immunoglobulin concentration was determined by using the photometric absorbance at 280 nm and 320 nm after buffer blank subtraction (UV-Vis spectrophotometer Evolution 500, Thermo Fisher Scientific, Waltham, USA). The absorbance at 320 nm was subtracted from the absorbance at 280 nm and this absorbance value was used to calculate the protein content according to the law of Lambert-Beer.

EXAMPLE 5

Conductivity and pH Monitoring

During the TFF process every time the concentration doubled 1 ml was taken from the retentate. The pH was determined by using the Microprocessor pH Meter pH 196 equipped with a pH single-rod measuring cell E50-1.5 from WTW (Weilheim, Germany). The conductivity was determined by using the ProfiLine Konduktumeter LF 197 equipped with a standard conductivity cell TetraCon **325** from WTW (Weilheim, Germany). All samples were tempered in a water bath to 25° C. before measuring pH and conductivity.

EXAMPLE 6

Size Exclusion High Pressure Liquid Chromatography

Size exclusion high pressure liquid chromatography (SE-HPLC) experiments were conducted with a TSK 3000 SWXL column (Tosoh Bioseparation GmbH, Stuttgart, Germany) on a Summit HPLC-system (Dionex, Idstein, Germany). The elution peaks were monitored at 280 nm by the UV diode array detector UVD170U from Dionex (Idstein, Germany). Isocratic chromatography was conducted at room temperature using an aqueous buffer composed of 200 mM potassium phosphate and 250 mM potassium chloride at pH 7.0 and a flow rate of 0.5 ml/min. Each sample contained 100 µg immunoglobulin load per injection. The chromatograms were integrated manually by using the Chromeleon software (Dionex, Idstein, Germany). Percentage of higher molecular weight species (HMWs) including dimers and larger soluble oligomers was determined as relative area (mAU*min.) referred to total area of the two HMW peaks, the monomer peak and the peak of lower molecular weight species (LMWs).

EXAMPLE 7

Histidine Assay

Every time the protein concentration in the retentate doubled the histidine concentration was determined. The samples were diluted to about 100 µM histidine concentration with purified water (Milli-Q, Millipore, Billerica, USA). Afterward, 500 µl diluted sample was mixed with 500 µl perchloric acid (5%) (Fluka, Steinheim, Germany). After 10 min. the sample was centrifuged at 25° C. with 13.000 rpm for 10 min. (miniSpin, Eppendorf, Hamburg, Germany). 100 µl of the supernatant were injected on a MonoS 5/50 GL CEX column (GE Healthcare, Uppsala, Sweden). Chromatographic runs were conducted on a Ultimate 3000 HPLC-system (Dionex, Idstein, Germany) at room temperature using a gradient elution applying two aqueous buffers composed of 50 mM acetate, pH 3.2 (buffer A) and 50 mM acetate, pH 3.2, 1 M sodium chloride (buffer B). A flow-rate of 1.0 ml/min. was applied. Elution was monitored at 210 nm. The chromatograms were integrated manually by using the Chromeleon software (Dionex, Idstein, Germany). To quantify the amount of histidine, the area (mAU*min.) of the defined peak was compared with a standard curve (r^2=0.9998).

EXAMPLE 8

Acetate Assay

Every time the protein concentration in the retentate doubled the acetate concentration was determined. The samples were diluted to about 25 mM acetate concentration with purified water (Milli-Q, Millipore, Billerica, USA). Afterwards, 500 µl diluted sample was mixed with 500 µl perchloric acid (5%) (Fluka, Steinheim, Germany). After 10 min. the sample was centrifuged at 25° C. with 13.000 rpm for 10 min. (miniSpin, Eppendorf, Hamburg, Germany). 100 µl of the supernatant were injected on a LiChrosorb RP C18 4/250 column (Merck KG, Darmstadt, Germany). Chromatographic runs were conducted on a Ultimate 3000 HPLC-system (Dionex, Idstein, Germany) at room temperature using a isocratic elution over 15 min. applying a aqueous buffer composed of 6 mM phosphoric acid (Fluka, Steinheim, Germany), pH 2.6 (Kordis-Krapez, M., et al., Food Technol. Biotechnol. 39 (2001) 93-99). After each sample run the column was washed with 3 ml acetonitrile (Merck KG, Darmstadt, Germany) to avoid sample carryover. A flow-rate of 1.0 ml/min. was applied. Elution was monitored at 210 nm. The chromatograms were integrated manually by using the Chromeleon software (Dionex, Idstein, Germany). To quantify the amount of acetate the area (mAU*min) of the defined peak was compared with a standard curve (r^2=0.9999).

EXAMPLE 9

Chloride Assay

Every time the protein concentration in the retentate doubled the chloride concentration was determined. The

samples were diluted 1:200 with purified water (Milli-Q, Millipore, Billerica, USA). 10 μl of the diluted sample was injected on an IonPac AS1 1-HC 2/250 column (Merck KG, Darmstadt, Germany). Chromatographic runs were conducted on a ICS 3000 Reagent-Free Ion Chromatography system (Dionex, Idstein, Germany) at room temperature using a gradient elution over 30 min. applying a aqueous solution up to 100 mM sodium hydroxide. A flow-rate of 0.38 ml/min. was applied. Detection was carried out on an ICS 3000 CD conductivity detector. The chromatograms were integrated manually by using the Chromeleon software (Dionex, Idstein, Germany). To quantify the amount of chloride the area (mAU*min) of the defined peak was compared with a standard curve (r^2=0.9963).

EXAMPLE 10

Sodium Assay

To quantify the amount of sodium ions, the samples were analyzed with the multi sensor system BioProfile100plus (NOVA Biomedical, Waltham, USA). Sodium ions were quantified at 25° C. after a two point calibration. Samples were diluted 1:1 with purified water (Milli-Q, Millipore, Billerica, USA) before measuring.

EXAMPLE 11

Turbidity Measurement

Turbidity was determined as photometric absorbance of the undiluted concentrates at 350 nm and 550 nm after buffer-blank subtraction, where no intrinsic chromophores of the monoclonal immunoglobulin absorb (UV-Vis spectrophotometer Evolution 500, Thermo Fisher Scientific, Waltham, USA) (Capelle, M. A. H., et al., Eur. J. Pharm. Biopharm. 65 (2007) 131-148). The samples were mixed before measuring. In the presence of suspended particles an increase in UV absorbance at all weave lengths occurs due to scattering effects (Eberlein, G. A., et al., PDA J of Pharmac. Science and Technol. 48 (1994) 224-230).

EXAMPLE 12

Light Obscuration

Light obscuration (LO) was used to monitor the formation of particles in a range of 1-200 μm similar to the method <788> Particulate Matter of Injection in the United States Pharmacopoeia and the European Pharmacopoeia method 2.9.1. (European Directorate for the Quality of Medicine (Ed.), European Pharmacopoeia, Deutscher Apotheker Verlag/Govi-Verlag, Stuttgart/Eschborn, 2001a, 140-141; United States Pharmacopeia Convention (Ed.), United States Pharmacopeia, United States Pharmacopeia Convention, Rockville, Md., 2002, 2046-2051). The particle counter SVSS-C (PAMAS Partikelmess- and Analysesysteme, Rutesheim, Germany) was equipped with a laser diode and a photodiode detector in order to determine the residual photocurrent after particles have passed the course of the beam. As particle sensor the HCB-LD-25/25 was applied. Concentrates of a higher content of particles than 120 000/ml where diluted with buffer to match the specified capacity of the used sensor. Three measurements of a volume of 0.5 ml for each sample were analyzed after an equilibrating flush of 0.3 ml. Results were calculated as mean value of three measurements and referred to a sample volume of 1.0 ml. Before diluting the concentrates the buffer was filtered using Stericup Express plus 0.1 μm filter devices (Millipore, Billerica, USA) and the particle burden was determined as described before.

EXAMPLE 13

Zeta Potential Measurement

To determine the charge of the protein at different pH values, electrophoretic mobility of the protein was determined by performing Laser-Doppler-Velocimetry using the Malvern Zetasizer Nano S (Malvern Instruments, Worcestershire, UK). The zeta potential ζ was calculated from the Henry's equation with assumption of uniform charge distribution by using the Malvern DTS software (Version 5.0, Malvern Instruments, Worcestershire, UK):

$$\mu_e = \frac{2\varepsilon k \zeta}{3\eta} \quad \text{(equation 4)}$$

Where μ_e is the electrophoretic mobility, ∈ is the dielectric constant of the solution, k_s is the model based constant with a value of 1.5 for a salt concentrations higher than 1 mM, η is the viscosity of the solution and ζ is the zeta potential. For sample preparation the 5 mg/ml mAb solutions were dialyzed into 50 mM acetate buffer pH 5.0 and titrated to a pH of 2.0 afterwards by using 0.2 M hydrochloric acid. The samples were titrated with a 0.2 M sodium hydroxide solution from pH 2 to pH 12 by applying the titrator MPT2 (Malvern Instruments, Worcestershire, UK). The zeta potential was determined in 15 steps between pH 2 and 12 in a temperature controlled folded capillary cell (Malvern Instruments, Worcestershire, UK) at 25° C. Each measurement was repeated threefold and mean values±SD are reported. See FIG. **9** for an exemplary zeta potential determination of an anti-IL-1R antibody.

EXAMPLE 14

Determination of Protein Solution Density

The density ρ of the protein solutions was determined at every protein concentration step. A pycnometer (Schott, Mainz, Germany) with a volume of 2.076 ml was filled with the sample solution previously tempered to 20° C. The mass of the unfilled and filled pycnometer was determined by using an analytical balance (MC 210 S, Sartorius, Göttingen, Germany). The density was calculated according to the common equation ϱ=m/V.

The invention claimed is:

1. A method for concentrating an immunoglobulin solution comprising the following steps:

a) providing an immunoglobulin solution with a pH value, with a first immunoglobulin protein concentration, and with a first concentration S^+ or S^- of a buffer substance,

b) adjusting said first concentration of said buffer substance to a second concentration S' and maintaining said pH value, whereby said second concentration S' is calculated with equation 2 if said buffer substance is a cation/neutral pair or with equation 3 if said buffer substance is a neutral/anion pair,

c) concentrating the solution of b) with a tangential flow filtration to a second immunoglobulin protein concentration,

wherein equation 2 is

$$S^+ = \frac{-zP + \sqrt{(zP)^2 + 4\left(\frac{S'}{\rho'}\left(\rho - \frac{PM_P}{1000}\right)\right)^2}}{2}$$

and equation 3 is

$$S^- = \frac{zP + \sqrt{(zP)^2 + 4\left(\frac{S'}{\rho'}\left(\rho - \frac{PM_P}{1000}\right)\right)^2}}{2}$$

with

the molar concentration in the retentate of positively/negatively charged solutes (S^+/S^-), the charge of the protein (z), the molar concentration (P) and the molecular weight (M_P) of the protein, the density of the solution in the retentate (ρ) and the permeate (ρ'), and the theoretical molar concentration of the diffusible solute (S').

2. The method of claim **1**, wherein said buffer substance is histidine and that said second concentration is calculated with equation 2.

3. The method of claim **1**, wherein said first concentration is approximately 20 mM.

4. The method of claim **3**, wherein said second concentration is of from 24 mM to 37 mM.

5. The method of claim **1**, wherein wherein said first concentration is approximately 46 mM.

6. The method of claim **5**, wherein said second concentration is of from 52 mM to 72 mM.

7. The method of claim **1**, wherein said buffer substance is acetate and that said concentration is calculated with equation 3.

8. The method of claim **7**, wherein said first concentration is 20 mM.

9. The method of claim **8**, wherein said second concentration is of from 8 to 19 mM.

10. The method of claim **7**, wherein said first concentration is 45 mM.

11. The method of claim **10**, wherein said second concentration is of from 41 mM to 48 mM.

12. The method of claim **1**, wherein the immunoglobulin is an anti-P selectin antibody or an anti-Aβ antibody.

13. A method for producing an immunoglobulin comprising

a) cultivating a cell comprising a nucleic acid encoding said immunoglobulin,

b) recovering said immunoglobulin from the cultivation medium or the cell of step a)

c) purifying said immunoglobulin,

d) concentrating said immunoglobulin with the method of claim **1** and thereby producing an immunoglobulin.

* * * * *

EXHIBIT LL

US009047438B2

(12) **United States Patent**
Belousov et al.

(10) **Patent No.: US 9,047,438 B2**
(45) **Date of Patent: Jun. 2, 2015**

(54) **CHROMATOGRAPHY EQUIPMENT CHARACTERIZATION**

(71) Applicant: **Hoffmann-La Roche Inc.**, Nutley, NJ (US)

(72) Inventors: **Anton Belousov**, Penzberg (DE); **Thomas Dams**, Pullach (DE); **Benjamin Gerwat**, Weilheim (DE)

(73) Assignee: **Hoffmann-La-Roche, Inc.**, Little Falls, NJ (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 139 days.

(21) Appl. No.: **13/922,101**

(22) Filed: **Jun. 19, 2013**

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(30) **Foreign Application Priority Data**

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G06F 19/00 (2011.01)
(Continued)

(52) **U.S. Cl.**
CPC ***G06F 19/70*** (2013.01); *G01N 30/50* (2013.01); ***G01N 30/86*** (2013.01); ***G01N 30/8665*** (2013.01); ***G01N 30/88*** (2013.01); ***C07K 1/22*** (2013.01)

(58) **Field of Classification Search**
CPC ... G01N 30/86; G01N 30/88; G01N 30/8665; G01N 30/50; G06F 19/70; C07K 1/22
USPC 210/635, 656, 657, 659, 143, 198.2; 530/413; 702/30, 81, 84, 104
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,677,171 A 10/1997 Hudziak et al.
5,821,337 A 10/1998 Carter et al.
(Continued)

FOREIGN PATENT DOCUMENTS

WO 98/46623 10/1998
WO WO 01/87329 A1 11/2001
(Continued)

OTHER PUBLICATIONS

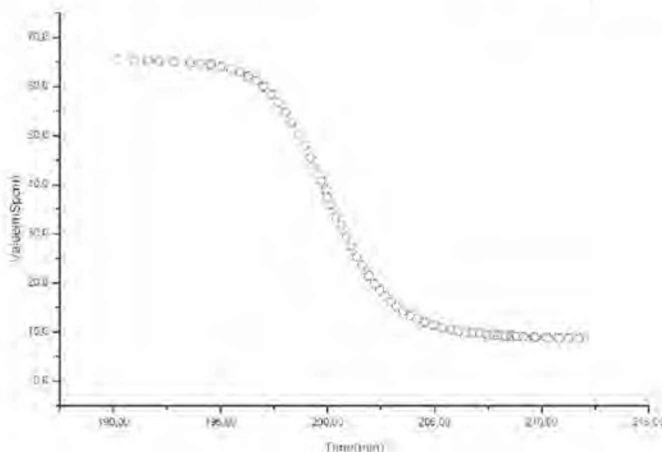
Sarker et al., "Study of the packing behavior of axial compression columns for preparative chromatography" Journal of Chromatography A 702:27-44 (1995).
(Continued)

Primary Examiner — Ernest G Therkorn
(74) *Attorney, Agent, or Firm* — Janet M. Martineau

(57) **ABSTRACT**

Herein is reported a method for determining whether a re-usable chromatography column packing, which is used at least for the second time in a purification step of a purification of a polypeptide, has reduced separation efficacy in said purification step of said purification of said polypeptide, comprising the following steps: a) identifying and determining the experimental data of an inert change of at least one physicochemical parameter of a mobile phase passing through said re-usable chromatography column packing, b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use, c) determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b), d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference, e) determining reduced separation efficacy of said re-usable chromatography column packing when the absolute value of the difference calculated in step d) is more than 0.1.

14 Claims, 9 Drawing Sheets



(51) **Int. Cl.**

G01N 30/88	(2006.01)
C07K 1/22	(2006.01)
G01N 30/50	(2006.01)

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,027,650 A	2/2000	Van Reis et al.	
8,357,301 B2 *	1/2013	Belousov et al.	210/656
2010/0004907 A1	1/2010	Kidal et al.	

FOREIGN PATENT DOCUMENTS

WO	2008/028974	3/2008
WO	2009/010271	1/2009
WO	2009/094203	7/2009
WO	2010/019814	2/2010
WO	2010/149367	12/2010

OTHER PUBLICATIONS

Guiochon et al. Fundamentals of Preparative and Nonlinear Chromatography 2nd edition edition. San Diego (USA):Elsevier Inc., (2006).

Larson et al., "Use of Process Data to Assess Chromatographic Performance in Production-Scale Protein Purification Columns" Biotechnol 19(2):485-492 (2003).

Moscariello et al., "Characterizing the performance of industial-scale columns" Journal of Chromatography A 908(1-2):131-141 (Jan. 26, 2001).

Vink et al., "Resolution and Column Efficiency in Chromatography" Journal of Chromatography 69:237-242 (1972).

Norling et al., "Impact of multiple re-use of anion-exchange chromatography media on virus removal" Journal of Chromatography A 1069:79-89 (2005).

Teeters et al., "Evaluating and monitoring the packing behavior of process-scale chromatography columns" Journal of Chromatography A 1069:43-64 (2005).

Bird et al., "Single-Chain Antigen-Binding Proteins" Science 242:423-426 (1988).

Hood et al. Immunology 2nd edition,Benjamin, NY,:2 pages (1984).

Hunkapiller et al., "The Growing Immunoglobulin Gene Superfamily" Nature 323:15-16 (1986).

Huston et al., "Protein Engineering of Antibody Binding Sites: Recovery of Specific Activity in an Anti-digoxin Single-Chain Fv Analogue Produced in *Escherichia coli*" P.N.A.S. USA 85:5879-5883 (1988).

Vijayalakshmi. "Antibody purification methods" Appl Biochem Biotech 75:93-102 (1998).

* cited by examiner

Fig. 1

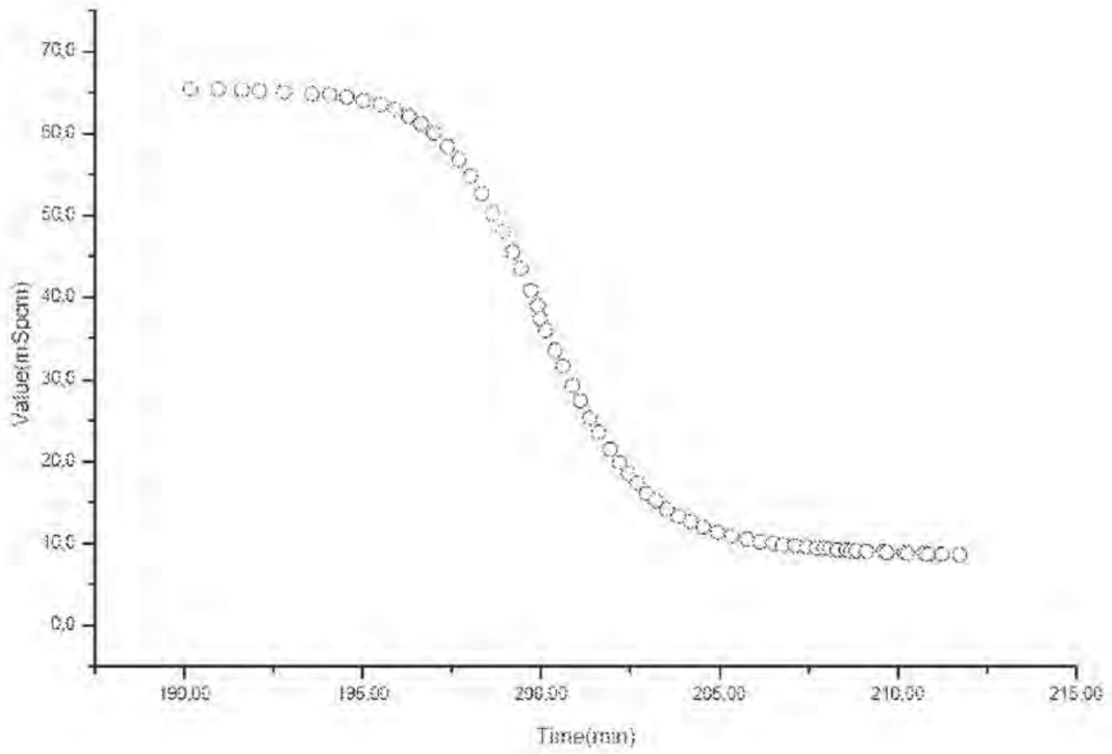


Fig. 2

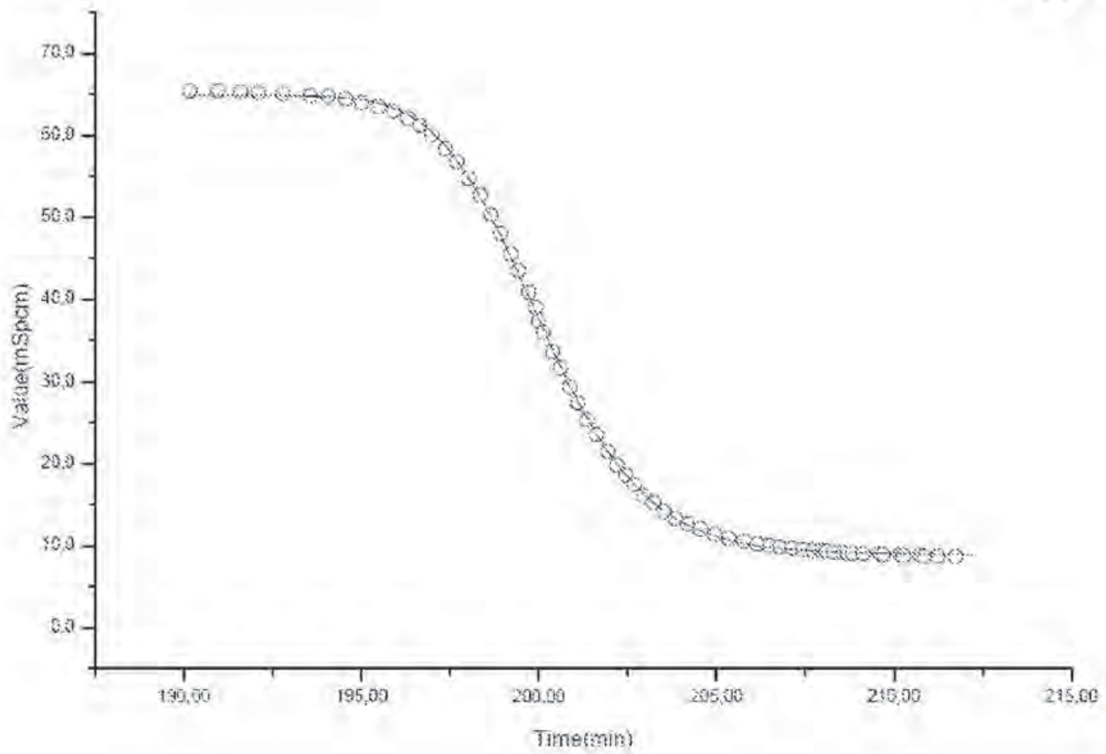


Fig. 3

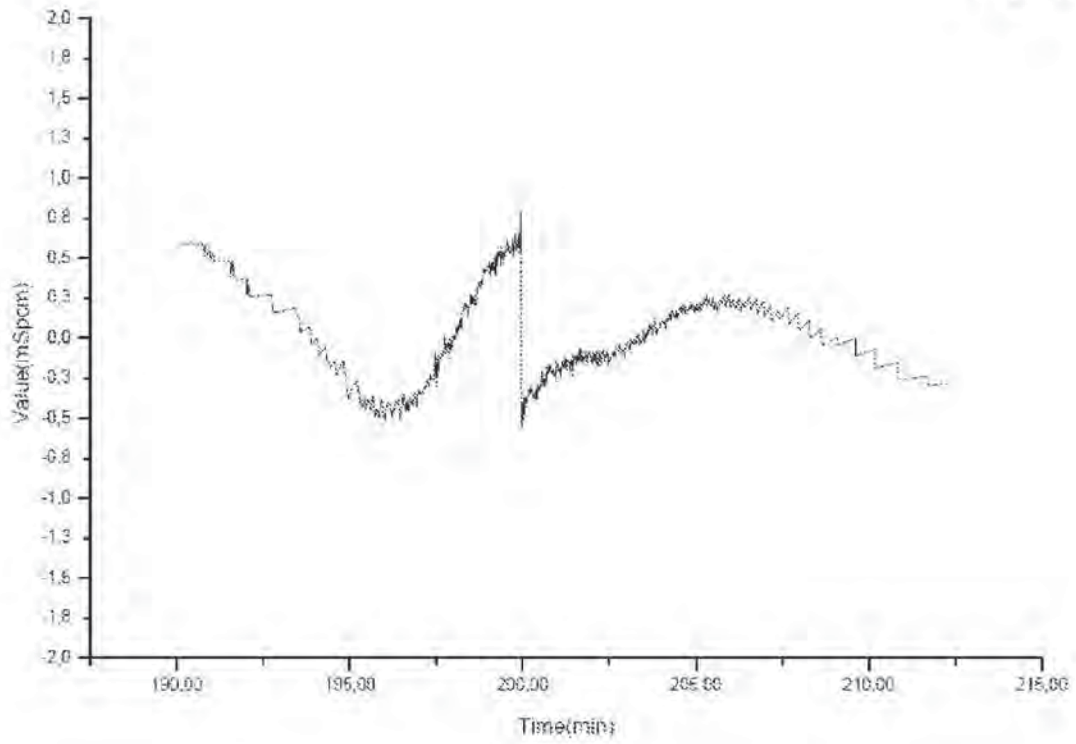


Fig. 4

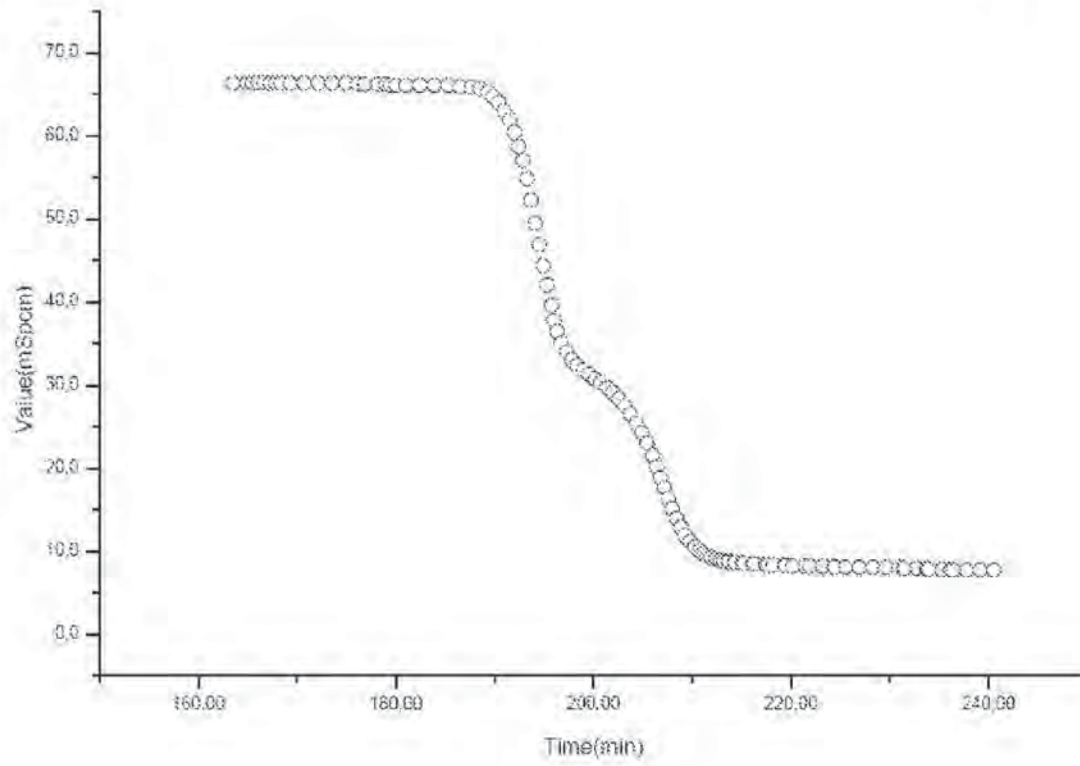


Fig. 5

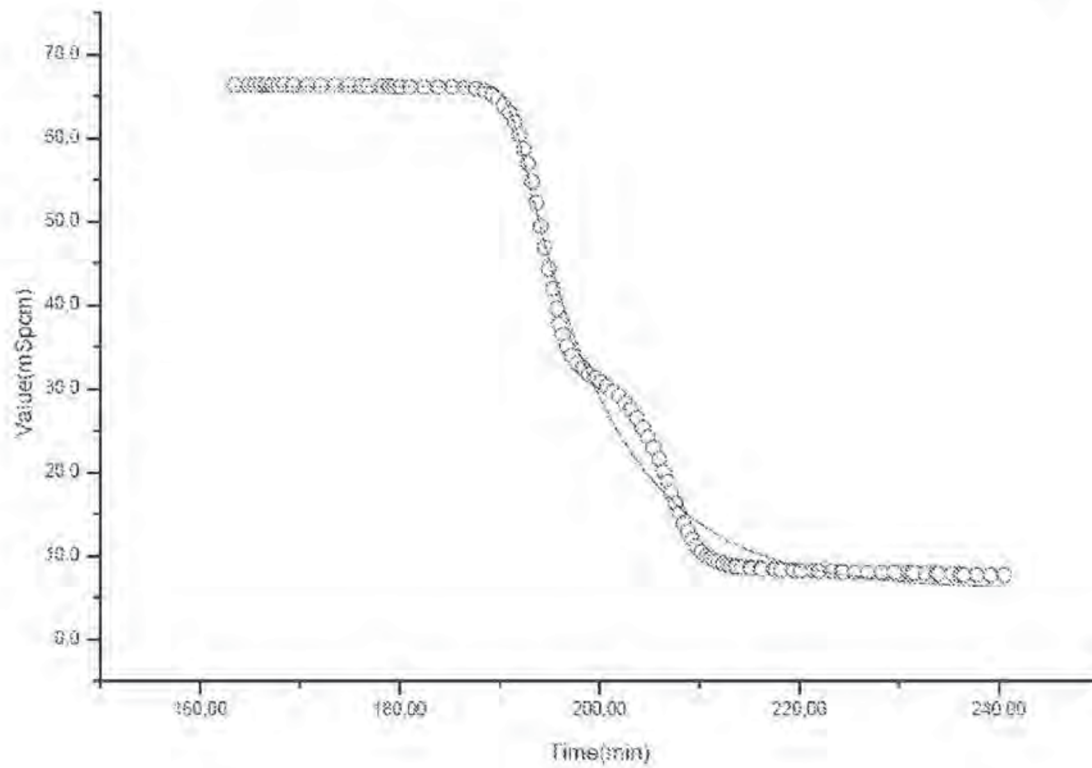


Fig. 6

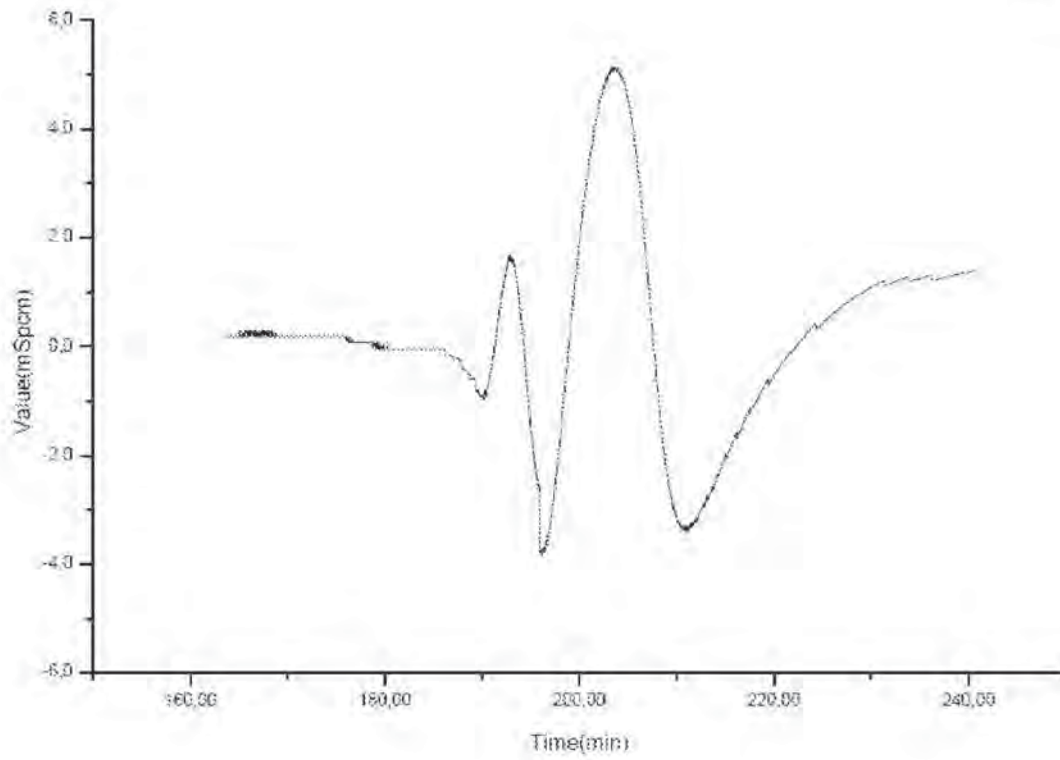


Fig. 7

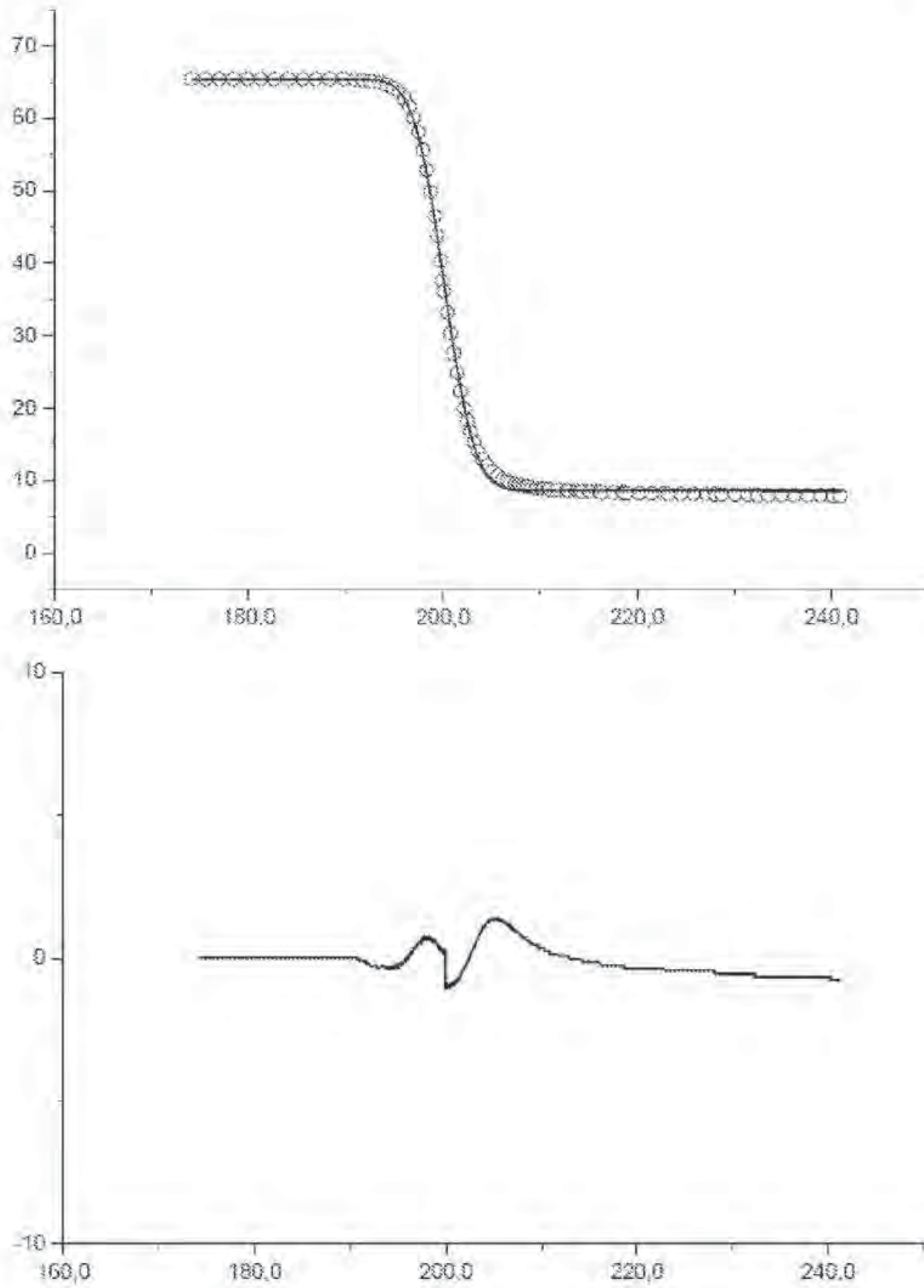


Fig. 8

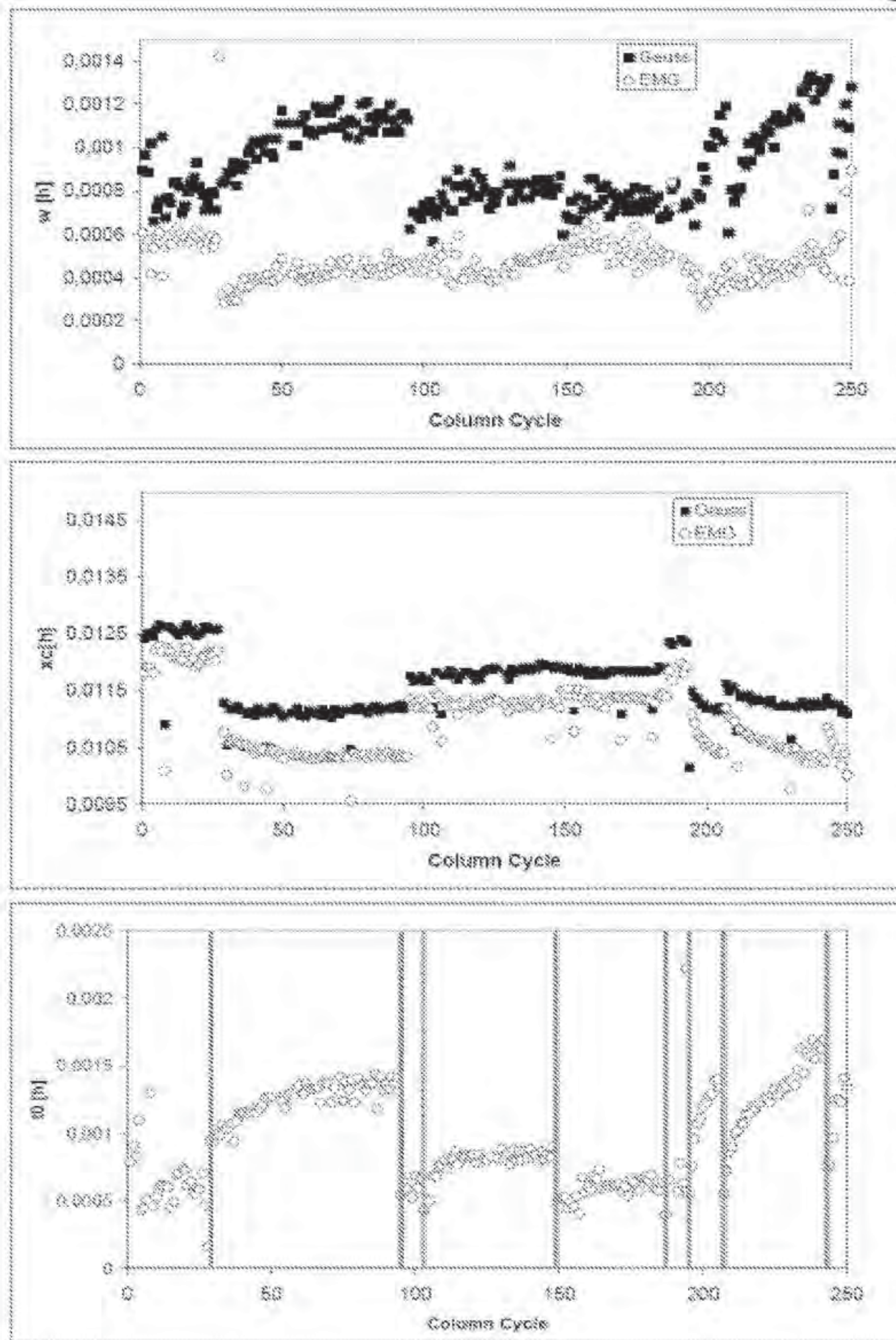
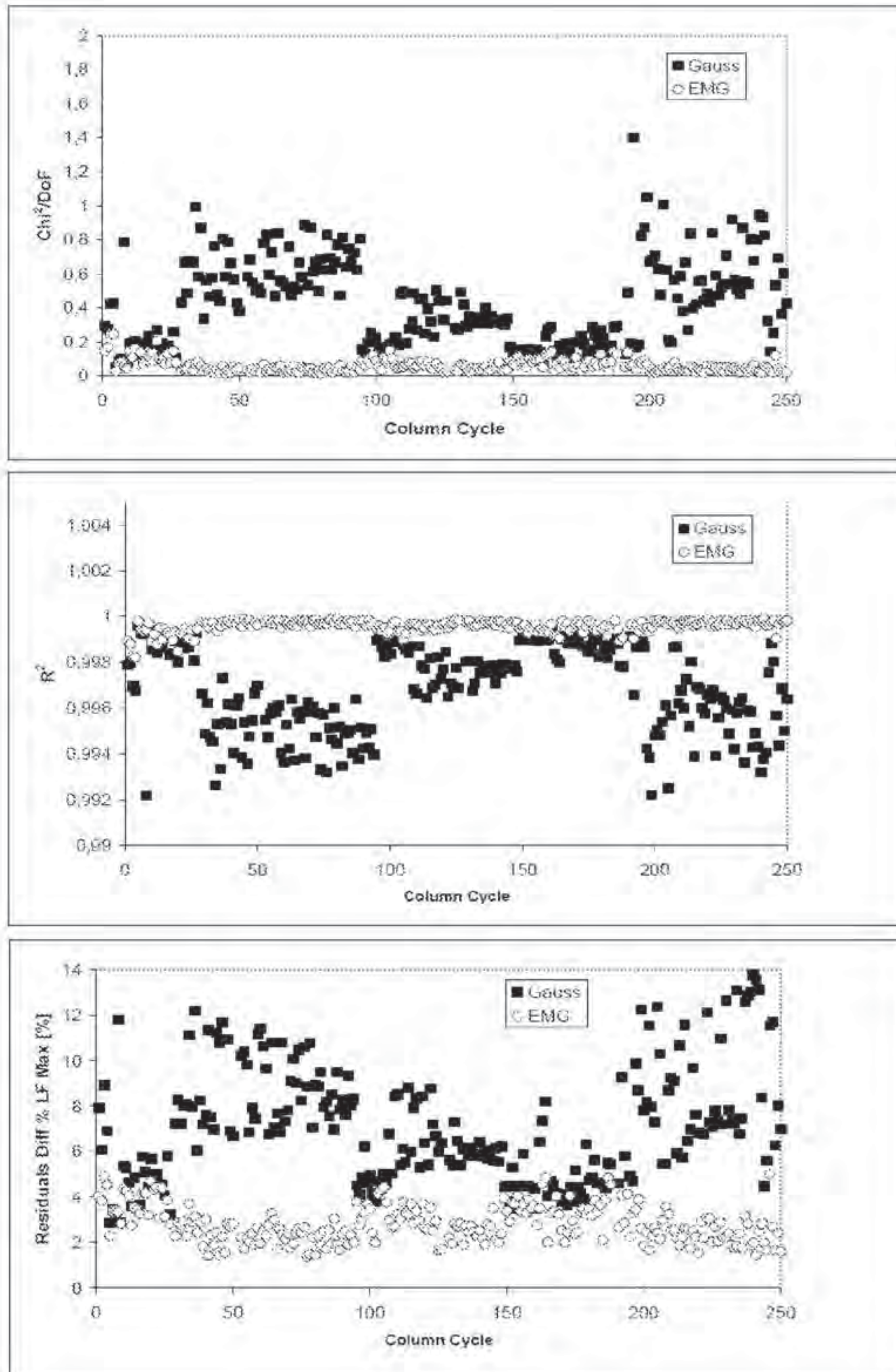


Fig. 9



Case 1:18-cv-00095-CFC Document 1-3 Filed 01/12/18 Page 609 of 681 PageID #: 1478

CHROMATOGRAPHY EQUIPMENT CHARACTERIZATION

This application is a continuation of International Patent Application No. PCT/EP2011/073243, filed Dec. 19, 2011, which is hereby incorporated herein by reference in its entirety and which claims priority benefit to EP 10196288.4 filed Dec. 21, 2010.

The herein reported method is in the field of chromatography, especially in the field of preparative column chromatography. It is herein reported a method for the direct determination of the quality of the packing of a chromatography column based on in process data. With this method a saving in process time and resources can be achieved as an additional data acquisition solely for the purpose of column integrity determination can be eliminated.

BACKGROUND OF THE INVENTION

Today almost all polypeptides used in medicaments are prepared recombinantly. Due to strict regulatory guidelines and requirements, by-products have to be removed from the therapeutic polypeptide preparation as much as possible. Therefore, at least one chromatography step is employed in down stream processing of the bulk raw polypeptide after recombinant production. As the dimension of the chromatography equipment with respect to the yield of the fermentation process, especially the separation capacity of chromatography columns, is limited, a multitude of batches have to be processed in order to be able to provide the required amount of purified therapeutic polypeptide.

To ensure that each batch of the purified therapeutic polypeptide has the same pharmaceutical effect, a list of analytical parameters has to be fulfilled for each batch. This can only be achieved if the steps of the purification process operate consistently and efficiently. But, if one step of the purification process does not work properly the obtained product will most probably not pass the analytical tests and, in the worst case, this batch cannot be used. Therefore, it is necessary to provide methods for determining the performance and efficacy of purification steps.

Teeters, M. A. and Quinones-Garcia, I. (J. Chrom. A 1069 (2005) 53-64) report the evaluating and monitoring the packing behavior of process-scale chromatography columns by using the responses to conductivity-based pulse and step inputs derived from tracer experiments and in-process transitions, especially from measured residence time distributions. Norling, et al. (Norling, L., et al., J. Chrom. A 1069 (2005) 79-89) report the impact of multiple re-use of anion-exchange chromatography media on virus removal. The use of process data to assess chromatographic performance in production-scale protein purification columns is reported by Larson, et al. (Larson, T. M., et al., Biotechnol. Prog. 19 (2003) 485-492). Moscariello, J., et al., J. Chrom. A 908 (2001) 131-141 report the characterization of the performance of industrial-scale columns. The resolution and column efficiency in chromatography is reported by Vink, H., J. Chrom. 69 (1972) 237-242. Sarker, M. and Guiochon, G., J. Chrom. A 702 (1995) 27-44 report a study of the packing behavior of axial compression columns for preparative chromatography.

The use of an integrated form of the Gaussian distribution function allows for the description of the packed bed characteristics, whilst neglecting effects outside the packed bed itself, which potentially influence the evaluation (see e.g. PCT/EP2010/003813). The implementation of equipment characteristics in the evaluation of non-Gaussian distributions observed during the assessment of packed chromatographic beds has been described by Guiochon, G., et al. (FUNDAMENTALS OF PREPARATIVE AND NONLINEAR CHROMATOGRAPHY; Guiochon, G., et al. (eds), Elsevier Inc., San Diego (USA), 2nd edition (2006)).

SUMMARY OF THE INVENTION

With the method as reported herein a determination of the decrease in the separation efficacy and/or packing quality of a re-usable chromatography column packing can be determined without the need to use and inject a further tracer compound prior to the separation of the crude polypeptide solution for the determination of column material integrity or the need for historical data of this purification step.

The method reported herein allows for determination of packed matrix parameters separately and independently from contributions and effects of the equipment involved. This allows for the discrimination and/or allocation of separate physical effects and for a comprehensive characterization of the chromatography equipment used independent of the scale, such as in analytical scale and in industrial scale.

The first aspect as reported herein is a method for determining whether a re-usable chromatography column packing, which is used at least for the second time in a purification step of a multi-step purification process of a polypeptide, has reduced separation efficacy, e.g. compared to the separation efficacy when it was used for the first time in the same purification step of the same multi-step purification process of the same polypeptide, comprising the following steps:

a) identifying and determining the experimental data of an inert change of at least one physicochemical parameter of a mobile phase passing through said re-usable chromatography column packing,
b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use determined in step a),
c) determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use determined in step a) and the function of formula I with the parameters determined in step b),
d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference,
e) determining reduced separation efficacy of said re-usable chromatography column packing when the absolute value of the difference calculated in step d) is more than 0.1,

wherein the function of formula I is

$$yI = -\frac{1}{2}A\cdot\exp\left(-\frac{x}{t_0}\right)\cdot\left(\exp\left(\frac{2x_c t_0 + w^2}{2t_0^2}\right)\left(erf\left(\frac{\left(\frac{x-x_c}{w}-\frac{w}{t_0}\right)}{\sqrt{2}}\right)+1\right)-\exp\left(\frac{x}{t_0}\right)\cdot erf\left(\frac{x-x_c}{\sqrt{2}\cdot w}\right)\right)+y_0 \qquad \text{formula I}$$

with parameters A and y_0 describing the respective signal changes, x_c the mean value, and w the standard deviation of the underlying Gaussian distribution function, respectively, and parameter t_0 describing the time constant of the underlying exponential decay function, and

wherein the erf-function used is defined as

$$erf(x) = \frac{2}{\sqrt{\pi}} \sum_{0}^{\infty} \frac{(-1)^n x^{2n+1}}{(2n+1)n!}.$$

Another aspect as reported herein is a method for the chromatographic purification of a polypeptide, in which at least one chromatography step with a re-usable chromatography column packing is comprised, comprising the following steps:

a) identifying and determining the experimental data of an inert change of at least one physicochemical parameter of a mobile phase passing through said re-usable chromatography column packing,

b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use,

c) determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b),

d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference,

wherein the function of formula I is

$$yI = -\frac{1}{2}A \cdot \exp\left(-\frac{x}{t_0}\right) \left(\exp\left(\frac{2x_c t_0 + w^2}{2t_0^2}\right)\left(erf\left(\frac{\left(\frac{x-x_c}{w} - \frac{w}{t_0}\right)}{\sqrt{2}}\right)+1\right) - \exp\left(\frac{x}{t_0}\right)\cdot erf\left(\frac{x-x_c}{\sqrt{2}\cdot w}\right) \right) + y_0 \quad \text{formula I}$$

with parameters A and y_0 describing the respective signal changes, x_c the mean value, and w the standard deviation of the underlying Gaussian distribution function, respectively, and parameter t_0 describing the time constant of the underlying exponential decay function, and wherein the erf-function used is defined as

$$erf(x) = \frac{2}{\sqrt{\pi}} \sum_{0}^{\infty} \frac{(-1)^n x^{2n+1}}{(2n+1)n!}.$$

and

further using the re-usable chromatography column packing when the absolute value of the difference calculated in step d) is 0.06 or less, or

performing additional characterization of the purified polypeptide when the absolute value of the difference calculated in step d) is more than 0.06 but less than 0.2, or

changing the re-usable chromatography column packing when the absolute value of the difference calculated in step d) is 0.2 or more.

In one embodiment said inert change of at least one physicochemical parameter of said mobile phase passing through said re-usable chromatography column packing is a significant signal change effected by the change of the concentration

of a substance that does not interact with the re-usable column packing contained in said mobile phase. In another embodiment said determining the experimental data is a determining over time the experimental data of a physicochemical parameter of an inert change. In a further embodiment said inert change of at least one physicochemical parameter of a mobile phase passing through said re-usable chromatography column packing is a change of the mobile phase from 100% of a solution containing a denaturing agent to 100% of a solution not containing said denaturing agent or from 100% of a solution not containing said denaturing agent to 100% of a solution containing a denaturing agent. In another embodiment the denaturing agent is selected from sodium hydroxide, guanidinium chloride, urea, or organic solvent. In one embodiment said step c) is determining the differences between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b) for each experimental data point. In one embodiment said signal change is a change in conductivity or in adsorption at 280 nm. In a further embodiment said inert change is a sigmoid change. In still another embodiment said at least one physicochemical parameter is determined in the conditioning or regeneration step.

DETAILED DESCRIPTION OF THE INVENTION

Herein is reported a method for the determination of packed chromatography material matrix parameters separately and independently from contributions and effects of the equipment involved. It has been found that this allows for the discrimination and/or allocation of separate physical effects influencing the chromatographic separation and also for a comprehensive characterization of the chromatography equipment used independent of the scale, such as in analytical scale and in industrial scale.

The first aspect as reported herein is a method for determining whether a re-usable chromatography column packing, which is used at least for the second time in a purification step of a purification of a polypeptide, has reduced separation efficacy compared to the separation efficacy when it was used for the first time in the same purification step of the same purification of the same polypeptide, comprising the following steps:

a) identifying an inert change and determining over time the experimental data of a physicochemical parameter of an inert change from 100% of a solution containing a denaturing agent to 100% of a solution not containing said denaturing agent, or vice versa, of a mobile phase passing through said re-usable chromatography column packing after the at least first use of the chromatography column packing,

b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use obtained in a),

c) determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b),

d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference,

e) determining reduced separation efficacy of said re-usable chromatography column packing when the absolute value of the difference calculated in step d) is more than 0.06.

wherein the function of formula I is

$$yI = -\frac{1}{2}A \cdot \exp\left(-\frac{x}{t_0}\right) \left(\exp\left(\frac{2x_c t_0 + w^2}{2t_0^2}\right)\left(erf\left(\frac{\left(\frac{x-x_c}{w} - \frac{w}{t_0}\right)}{\sqrt{2}}\right) + 1\right) - \exp\left(\frac{x}{t_0}\right) \cdot erf\left(\frac{x-x_c}{\sqrt{2} \cdot w}\right) \right) + y_0 \quad \text{formula I}$$

with parameters A and y_0 describing the respective signal changes, xc the mean value and w the standard deviation of the underlying Gaussian distribution function, respectively, and parameter t0 describing the time constant of the underlying exponential decay function. Further, the erf-function used is defined as

$$erf(x) = \frac{2}{\sqrt{\pi}} \sum_{0}^{\infty} \frac{(-1)^n x^{2n+1}}{(2n+1)n!}.$$

It has been found that with the method as reported herein contributions of the packed chromatography material matrix and also the equipment surrounding the packed matrix can be used to assess the quality of a chromatography column packing as well as a column chromatography separation.

The term "re-usable chromatography column packing" denotes a chromatography material that is packed into a chromatography column whereby the chromatography material is obtained after a purification in a not modified form, i.e. with the same characteristics as prior to the purification. A purification step denotes in general a cycle comprising the conditioning of the chromatography column packing, the application of the crude polypeptide solution, optionally the washing of the chromatography material, the recovery of the purified polypeptide from the chromatography column packing and the regeneration of the chromatography column packing. In one embodiment of the aspects as reported herein the inert change of at least one physicochemical parameter of a mobile phase passing through said re-usable chromatography column packing is a change of a physicochemical parameter over time and/or in the conditioning of the chromatography column packing and/or in the regeneration of the chromatography column packing.

The definition of a re-usable chromatography column packing as outlined above requires that all individual steps of a purification are perfectly reversible. But this is not the case. During the purification step e.g. the completely homogeneous nature of the packed chromatography material may become disturbed and the flow through the separation matrix can be compromised. At one point in time the separation efficacy and/or recovery and/or packing quality of the re-usable chromatography material is still sufficient to allow for a purification of the polyp eptide from by-products but not in a purity fulfilling the requirements of the specification of said polypeptide. As a result this batch of the polypeptide maybe cannot be used as a therapeutic and has to be further treated or discarded.

With the method as reported herein a determination of the decrease in the separation efficacy and/or packing quality of and/or in the recovery from a re-usable chromatography column packing is possible without i) the need to use and inject a further tracer compound prior to the separation of the crude polypeptide solution for the determination of column material integrity or ii) the need for historical data of this purification step. Thus, the method according to the current invention allows for the determination of the quality of a re-usable chromatography column packing based on data that is generally obtained during the chromatography purification step of the polypeptide making additional steps such as tracer substance injection unnecessary.

The method as reported herein is based on the finding that an inert change of at least one physicochemical parameter of a mobile phase passing through a re-usable chromatography column packing during the purification of a polypeptide can be used for determining the chromatography material separation efficacy and/or packing quality. Such an "inert change" is the change of at least one, preferably one, physicochemical parameter over time, such as the concentration of a substance contained in the mobile phase, or of the mobile phase itself during the purification step. The substance does not interact with the functionality of the chromatography material effecting the purification of the polypeptide. Exemplary inert changes of at least one physicochemical parameter of a mobile phase passing through said re-usable chromatography column packing are i) a change from denaturing conditions to non-denaturing conditions, or ii) a change from strongly alkaline conditions to buffered conditions, or iii) a change from organic solvent to water. In one embodiment the change is from 100% of a 0.5 to 1 M sodium hydroxide solution or a 5 M guanidinium chloride solution or an 8 M urea solution or organic solvent to 100% buffer or 100% water, optionally comprising an ionizing agent such as trifluoro acetic acid in up to 1% (v/v). Or vice versa the changes is i) from non-denaturing conditions to denaturing conditions, or ii) from buffered conditions to strongly alkaline conditions, or iii) from water to organic solvent. In another embodiment the change is from 100% buffer or 100% water, optionally comprising an ionizing agent such as trifluoro acetic acid in up to 1% (v/v), to 100% of a 0.5 to 1 M sodium hydroxide solution, or to 100% of a 5 M guanidinium chloride solution, or to 100% of an 8 M urea solution or organic solvent. A chromatogram for an inert change showing no reduction in separation efficacy/packing quality is shown in FIG. **1** and a chromatogram for an inert change showing a reduction in separation efficacy/packing quality is shown in FIG. **4**.

In one embodiment the inert change of the at least one physicochemical parameter over time is determined by the experimental data recorded during the purification, such as the absorption at 280 mn, or the conductivity of the mobile phase leaving the chromatography column, or the organic solvent concentration leaving the chromatography column.

The term "mobile phase" denotes a liquid that is used in column chromatography and that surrounds the chromatography material of the chromatography column packing, which in turn is the stationary phase.

It has been found that from a comparison of the experimental data recorded during the inert change of the at least second use of said re-usable chromatography column packing with the recorded data during the inert change of the at least second use of said re-usable chromatography column packing fitted to the function of formula I a determination of the separation efficacy/packing quality of the re-usable chromatography column packing can be obtained. In one embodiment the experimental data is recorded during the at least second purification of said polypeptide with a column packing made with chromatography material that has been used one or more times in the same purification steps of the same purification of the same polypeptide prior to this use. The function of formula I is as follows:

$$yI = -\frac{1}{2}A\cdot\exp\left(-\frac{x}{t_0}\right)\left(\exp\left(\frac{2x_c t_0 + w^2}{2t_0^2}\right)\left(erf\left(\frac{\left(\frac{x-x_c}{w}-\frac{w}{t_0}\right)}{\sqrt{2}}\right)+1\right)-\exp\left(\frac{x}{t_0}\right)\cdot erf\left(\frac{x-x_c}{\sqrt{2}\cdot w}\right)\right)+y_0 \quad \text{formula I}$$

with absolute signal values determined by the solvent employed (A and y_0), the mean value and the standard deviation of the underlying Gaussian distribution function (x_c, w) the time constant of the underlying exponential decay function (t_0).

Further, the erf-function used is defined as

$$erf(x) = \frac{2}{\sqrt{\pi}}\sum_{0}^{\infty}\frac{(-1)^n x^{2n+1}}{(2n+1)n!}.$$

The methods as reported herein employ the integrated form of the exponentially modified Gaussian distribution yielding quality attributes for the packing of the column, i.e. values for x_c, w, corresponding to retention time and peak broadening of the packed matrix. Further to the parameters determined by the packed matrix equipment characteristics are accounted for by the exponential time constant t_0. With the function of formula I the deviation from a purely diffusion controlled distribution mechanism of a compound(s) contained in an approximately rectangular pulse of the compound(s) at the column inlet can be determined. Due to the fact, that e.g. by using industrial-scale equipment, the contribution of the equipment by extended mixing times can result in significant deviations from the rectangular pulse form, this has to be accounted for by employing the exponentially modified formula described above.

The determination of the separate effects, i.e. packed matrix quality and equipment characteristics is achieved by comparing the function of formula I fitted to the experimental data recorded during the identified inert change in the purification of the polypeptide with the recorded experimental data itself, whereby the difference during the inert change of the fitted function and the not-fitted experimental data should not exceed a predetermined threshold in order to provide a purified polypeptide of desired characteristics.

The comparison is done by calculating the difference between the experimental data with which the function of formula I has been fitted and the fitted function of formula I. In order to make the results of individual purifications comparable to each other the results or the difference are normalized, e.g. by dividing the value with the maximum value of the experimental data of said physicochemical parameter during said inert change. In one embodiment a normalized difference function, as shown in the following formula II, is used:

$$yII = y - \left(-\frac{1}{2}A\cdot\exp\left(-\frac{x}{t_0}\right)\left(\exp\left(\frac{2x_c t_0 + w^2}{2t_0^2}\right)\left(erf\left(\frac{\left(\frac{x-x_c}{w}-\frac{w}{t_0}\right)}{\sqrt{2}}\right)+1\right)-\exp\left(\frac{x}{t_0}\right)\cdot erf\left(\frac{x-x_c}{\sqrt{2}\cdot w}\right)\right)+y_0\right) \quad \text{formula II}$$

Thus, in one embodiment step b) reads: determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use and determining therewith also the parameters of a normalized difference function of formula II, and step c) reads: determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula II with the parameters determined in step b). In another embodiment said normalization is in the step of calculating the difference between the maximum value and the minimum value of the difference determined between the experimental data and the function of formula I fitted to said experimental data by normalizing said difference by dividing said difference with the maximum value of the experimental data of said physicochemical parameter during said inert change.

An exemplary difference function for a chromatogram for an inert change with no reduction in separation efficacy and/or packing quality is shown in FIG. **3** and for a chromatogram for an inert change with a reduction in separation efficacy and/or packing quality is shown in FIG. **6**.

For the calculation of the absolute difference value the global maximum and the global minimum of the difference between the experimental data and the fitted experimental data calculated for each experimental data point is determined. The difference between this maximum value and this minimum value is calculated and provides a parameter with which the packing quality of the re-usable chromatography column packing can be determined. In one embodiment said difference is normalized by dividing the calculated difference values with the maximum value of the experimental data used for the calculation.

Depending on the polypeptide to be purified and its characteristics to be achieved threshold values for the absolute difference between the maximum value and the minimum value of the difference function can be given whereby the individual exceeding of each of the threshold values results in an action to be performed. In one embodiment said difference between the maximum value and the minimum value of the normalized difference function can be accepted and the packing can be used further if the absolute value of the difference calculated is less than 0.2 or 0.1 or 0.06. In one embodiment said difference between the maximum value and the minimum value of the normalized difference can be accepted but additional analyses and/or assessments have to be performed to ensure the specification conformity of the purified polypeptide if the absolute value is 0.06 or more but less than 0.2, or 0.1 or more but less than 0.2, or 0.1 or more but less than 0.15. In one embodiment said difference between the maximum value and the minimum value of the normalized difference cannot be accepted and the packing has to be changed/renewed if the absolute value is 0.2 or more, or 0.15 or more.

Therefore, another aspect as reported herein is a method for the chromatographic purification of a polypeptide, wherein at least one chromatography step is contained which employs a re-usable chromatography column packing, characterized in that said method comprises the following steps:

a) identifying and determining the experimental data of an inert change of at least one physicochemical parameter of a mobile phase passing through said re-usable chromatography column packing,
b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use,
c) determining the difference between the experimental data of the inert change of the physicochemical param-

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eter of the at least second use and the function of formula I with the parameters determined in step b),

d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference,

wherein the function of formula I is

$$yI = -\frac{1}{2}A \cdot \exp\left(-\frac{x}{t_0}\right) \left(\exp\left(\frac{2x_c t_0 + w^2}{2t_0^2}\right)\left(erf\left(\frac{\left(\frac{x-x_c}{w} - \frac{w}{t_0}\right)}{\sqrt{2}}\right) + 1\right) - \exp\left(\frac{x}{t_0}\right) \cdot erf\left(\frac{x-x_c}{\sqrt{2} \cdot w}\right) \right) + y_0 \quad \text{formula I}$$

with absolute signal values determined by the solvent employed (A and y_0), the mean value and the standard deviation of the underlying Gaussian distribution function (x_c, w), the time constant of the underlying exponential decay function (t_0), and

wherein the erf-function used is defined as

$$erf(x) = \frac{2}{\sqrt{\pi}} \sum_{0}^{\infty} \frac{(-1)^n x^{2n+1}}{(2n+1)n!},$$

and

further using the re-usable chromatography column packing when the absolute value of the difference calculated in step d) is 0.06 or less, or

performing additional assessment and/or characterization of the purified polypeptide when the absolute value of the difference calculated in step d) is 0.06 or more but less than 0.2, or

changing the re-usable chromatography column packing when the absolute value of the difference calculated in step d) is 0.2 or more.

A "polypeptide" is a polymer consisting of amino acids joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 20 amino acid residues may be referred to as "peptide", whereas molecules consisting of two or more polypeptides or comprising one polypeptide of more than 100 amino acid residues may be referred to as "protein". A polypeptide may also comprise non-amino acid components, such as carbohydrate groups, metal ions, or carboxylic acid esters. The non-amino acid components may be added by the cell, in which the polypeptide is expressed, and may vary with the type of cell. Polypeptides are defined herein in terms of their amino acid backbone structure or the nucleic acid encoding the same. Additions such as carbohydrate groups are generally not specified, but may be present nonetheless.

In one embodiment said polypeptide is recombinantly produced. In another embodiment said polypeptide is an immunoglobulin or an immunoglobulin conjugate. The term "immunoglobulin" refers to a protein consisting of one or more polypeptide(s) substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the different constant region genes as well as the myriad immunoglobulin variable region genes. Immunoglobulins may exist in a variety of formats, including, for example, Fv, Fab, and $F(ab)_2$ as well as single chains (scFv) or diabodies (e.g. Huston, J. S., et al., Proc. Natl. Acad. Sci. USA 85 (1988) 5879-5883; Bird, R. E., et al., Science 242 (1988) 423-426; in general, Hood, L. E., et al., Immunology, Benjamin N.Y., 2nd edition (1984); and Hunkapiller, T. and Hood, L. E., Nature 323 (1986) 15-16). The term "immunoglobulin conjugate" denotes a polypeptide comprising at least one domain of an immunoglobulin heavy or light chain conjugated via a peptide bond to a further polypeptide. The further polypeptide is either a non-immunoglobulin peptide, such as a hormone, or growth receptor, or antifusogenic peptide, or complement factor, or the like, or an immunoglobulin fragment, such as Fv, Fab, and $F(ab)_2$ as well as single chain antibody (scFv) or diabody.

Methods for purifying polypeptides and immunoglobulins are well established and widespread used and are employed either alone or in combination. Such methods are, for example and in certain embodiments, affinity chromatography using microbial-derived proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange chromatography), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and preparative electrophoretic methods (such as gel electrophoresis, capillary electrophoresis) (Vijayalakshmi, M. A., Appl. Biochem. Biotech. 75 (1998) 93-102). In one embodiment said chromatography column packing is a chromatography material selected from an affinity chromatography material, or an ion exchange chromatography material, or a thiophilic adsorption chromatography material, or a hydrophobic interaction chromatography material, or an aromatic adsorption chromatography material, or a metal chelate affinity chromatography material, or a size exclusion chromatography material.

In another embodiment the method as reported herein is used for the determination if the process hardware except the chromatography material has reduced separation efficacy.

The following examples and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. As the polypeptides erythropoietin and an anti-HER2 antibody were available in sufficient quantities in our laboratory at the time the invention was made the invention is exemplified with these polypeptides. This should not be understood as limitation but only as examples of the invention. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

DESCRIPTION OF THE FIGURES

FIGS. **1** to **6** were derived using data from a chromatographic step employed for purification of erythropoietin.

FIGS. **7** to **9** were derived using data from a chromatographic step employed for purification of an anti-HER2 antibody.

FIG. **1** Experimental data for an exemplary inert change of the conductivity for a re-usable chromatography column packing with no reduced separation efficacy/packing quality (open circles); X-axis: time [min]; Y-axis: conductivity [mS/cm].

FIG. **2** Experimental data for an exemplary inert change of the conductivity for a re-usable chromatography column packing with no reduced separation efficacy/packing quality

(open circles) and the fitted function according to a function of formula I; X-axis: time [min]; Y-axis: conductivity [mS/cm].

FIG. **3** Absolute difference between the experimental data for an exemplary inert change of the conductivity for a re-usable chromatography column packing with no reduced separation efficacy/packing quality and the fitted function according to formula II; X-axis: time; Y-axis: difference.

FIG. **4** Experimental data for an exemplary inert change of the conductivity for a re-usable chromatography column packing with reduced separation efficacy/packing quality (open circles); X-axis: time [min]; Y-axis: conductivity [mS/cm].

FIG. **5** Experimental data for an exemplary inert change of the conductivity for a re-usable chromatography column packing with reduced separation efficacy/packing quality (open circles) and the fitted function according to a function of formula I; X-axis: time [min]; Y-axis: conductivity [mS/cm].

FIG. **6** Absolute difference between the experimental data for an exemplary inert change of the conductivity for a re-usable chromatography column packing with reduced separation efficacy/packing quality and the fitted function according to a function of formula II; X-axis: time [min]; Y-axis: difference [mS/cm].

FIG. **7** Fitting of conductivity data used in FIG. **1** to formula III employing Gaussian distribution without the contribution of equipment characteristics:

$$yIII = \frac{1}{2}P1 \cdot \left(1 + erf\left(\frac{x - x_c}{w \cdot \sqrt{2}}\right)\right) + A0 \quad \text{formula III}$$

Upper part: Data and fitted curve. lower part: difference curve of data from fitted curve.

X-axis: time [min]; Y-axis: conductivity [mS/cm].

FIG. **8** Monitoring of column integrity with a method as reported herein over 250 chromatographic cycles using formula I (depicted as EMG) and formula III (depicted as Gauss). Integrity parameters shown are x_c (retention time), w (peak broadening) and t_0 (equipment characteristics). In the figure for t_0 column repacks that became necessary due to decreased packing quality are indicated by vertical bars in light grey.

FIG. **9** Monitoring of column integrity with a method as reported herein over 250 chromatographic cycles using formula I (depicted as EMG) and formula III (depicted as Gauss). Quality of fit parameters shown are X^2 (chi-square), R^2 (R-factor) and residuals difference normalized to maximum conductivity as described for formula II.

EXAMPLE 1

Fermentation and Purification of Erythropoietin

Erythropoietin can be produced and purified e.g. according to WO 01/87329.

The purification comprises some chromatography steps. One of these is a Blue Sepharose chromatography. Blue Sepharose consists of Sepharose beads to the surface of which the Cibacron blue dye is covalently bound. Since erythropoietin binds more strongly to Blue Sepharose than most non-proteinaceous contaminants, some proteinaceous impurities and PVA, erythropoietin can be enriched in this step. The elution of the Blue Sepharose column is performed by increasing the salt concentration as well as the pH. The column is filled with Blue Sepharose, regenerated with NaOH and equilibrated with equilibration buffer (sodium/calcium chloride and sodium acetate). The acidified and filtered fermenter supernatant is loaded. After completion of the loading, the column is washed first with a buffer similar to the equilibration buffer containing a higher sodium chloride concentration and consecutively with a TRIS-base buffer. The product is eluted with a TRIS-base buffer and collected in a single fraction in accordance with the master elution profile.

During the equilibration, separation, and regeneration step of the chromatography cycle the conductivity of the mobile phase at the outlet of the column is determined and recorded with a standard conductivity measuring device.

Fermentation and Purification of an Anti-HER2 Antibody

An anti-HER2 antibody can be produced and purified e.g. according to U.S. Pat. No. 5,821,337 or U.S. Pat. No. 5,677,171.

Antibody in harvested cell culture fluid samples can be captured and purified using a specific affinity chromatography resin. Protein A resin (Millipore, Prosep-vA High Capacity) was selected as the affinity resin for antibody purification. The resin was packed in a column.

The resin was exposed to buffers and harvested cell culture fluid (HCCF) at a linear flow rate between 260-560 cm/hr. The resin was equilibrated with 25 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 7.1.

For each purification, the resin was loaded between 5-15 mg antibody per mL of resin. After loading, the resin was washed with 25 mM Tris, 25 mM NaCl, 5 mM EDTA, 0.5 M TMAC, pH 5, and then the antibody was eluted using 0.1 M citric acid, pH 2.8. Elution pooling was based on UV absorbance at 280 mn measured inline after the column. The purified elution pools were pH-adjusted using 1 M TRIS buffer to pH 5-6. After regeneration of the resin with 0.1 M guanidinium hydrochloride, the same or similar packed resins were used for subsequent purification of other HCCF solutions.

The antibody concentration in the purified protein A pool was measured using UV spectrometry at 280 mn. The purified protein A elution pools were analyzed by size exclusion chromatography to quantitate the percentage of intact antibody at 150 kDa molecular weight.

EXAMPLE 2

Change in Column Properties

The column can be monitored over the process continuously using the method as reported herein. Subtle changes become detectable independently of changes of other process parameters. In FIGS. **8** and **9** changes in the column packing are shown over the complete lifetime of the Prosep A columns used in the purification of an anti-HER2 antibody. In those cases, where repacking became necessary, there is a clear indication of changes in the parameters derived from packed bed quality (see. FIG. **8**).

The invention claimed is:

1. A method for determining whether a re-useable chromatography column packing, which is used at least for the second time in a purification step of a purification of a polypeptide, has reduced separation efficacy in said purification step of said purification of said polypeptide, characterized in that said method comprises the following steps:

a) identifying and determining the experimental data of an inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing of the at least second use,

b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use,

c) determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b),

d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference,

e) determining reduced separation efficacy of said re-useable chromatography column packing when the absolute value of the difference calculated in step d) is more than 0.1,

wherein the function of formula I is

$$yI = -\frac{1}{2}A\cdot\exp\left(-\frac{x}{t_0}\right)\left(\exp\left(\frac{2x_c t_0 + w^2}{2t_0^2}\right)\left(erf\left(\frac{\left(\frac{x-x_c}{w}-\frac{w}{t_0}\right)}{\sqrt{2}}\right)+1\right)-\exp\left(\frac{x}{t_0}\right)\cdot erf\left(\frac{x-x_c}{\sqrt{2}\cdot w}\right)\right)+y_0 \quad \text{formula I}$$

with absolute signal values determined by the solvent employed (A and y_0), the mean value and the standard deviation of the underlying Gaussian distribution function (x_c, w), the time constant of the underlying exponential decay function (t_0), and

wherein the erf-function used is defined as

$$erf(x) = \frac{2}{\sqrt{\pi}}\sum_0^\infty \frac{(-1)^n x^{2n+1}}{(2n+1)n!}.$$

2. The method according to claim 1, characterized in that said inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing is a change effected by the change of the concentration of a substance in the mobile phase that does not interact with the re-useable column packing.

3. The method according to claim 1, characterized in that said inert change is a change in conductivity or in adsorption at 280 mn.

4. The method according to claim 1, characterized in that said inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing is a change of from 100% of a solution containing a denaturing agent to 100% of a solution not containing said denaturing agent, or vice versa.

5. The method according to claim 4 characterized in that said denaturing agent is selected from sodium hydroxide, guanidinium chloride, urea or organic solvent.

6. The method according to claim 1, characterized in that said inert change is a sigmoid change.

7. The method according to claim 1, characterized in that said inert change is a change over time.

8. A method for the chromatographic purification of a polypeptide, wherein at least one chromatography step using a re-useable chromatography column packing is contained, characterized in that said method comprises the following steps:

a) identifying and determining the experimental data of an inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing of the at least second use,

b) determining the parameters of a function of formula I by fitting the experimental data of the inert change of the physicochemical parameter of the at least second use,

c) determining the difference between the experimental data of the inert change of the physicochemical parameter of the at least second use and the function of formula I with the parameters determined in step b),

d) calculating the difference between the maximum value and the minimum value of the difference determined in step c) and normalizing said difference,

wherein the function of formula I is

$$yI = -\frac{1}{2}A\cdot\exp\left(-\frac{x}{t_0}\right)\left(\exp\left(\frac{2x_c t_0 + w^2}{2t_0^2}\right)\left(erf\left(\frac{\left(\frac{x-x_c}{w}-\frac{w}{t}\right)}{\sqrt{2}}\right)+1\right)-\exp\left(\frac{x}{t_0}\right)\cdot erf\left(\frac{x-x_c}{\sqrt{2}\cdot w}\right)\right)+y_0 \quad \text{formula I}$$

with absolute signal values determined by the solvent employed (A and y_0), the mean value and the standard deviation of the underlying Gaussian distribution function (x_c, w), the time constant of the underlying exponential decay function (t_0), and

wherein the erf-function used is defined

$$erf(x) = \frac{2}{\sqrt{\pi}}\sum_0^\infty \frac{(-1)^n x^{2n+1}}{(2n+1)n!},$$

and

further using the re-useable chromatography column packing when the absolute value of the difference calculated in step d) is 0.06 or less, or

performing an additional characterization and/or assessment of the purified polypeptide when the absolute value of the difference calculated in step d) is more than 0.06 but less than 0.2, or

changing the re-useable chromatography column packing when the absolute value of the difference calculated in step d) is 0.2 or more.

9. The method according to claim 8, characterized in that said inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing is a change effected by the change of the concentration of a substance in the mobile phase that does not interact with the re-useable column packing.

10. The method according to claim 8, characterized in that said inert change is a change in conductivity or in adsorption at 280 mn.

11. The method according to claim 8, characterized in that said inert change of at least one physicochemical parameter of a mobile phase passing through said re-useable chromatography column packing is a change of from 100% of a solution containing a denaturing agent to 100% of a solution not containing said denaturing agent, or vice versa.

12. The method according to claim 11, characterized in that said denaturing agent is selected from sodium hydroxide, guanidinium chloride, urea or organic solvent.

13. The method according to claim **8**, characterized in that said inert change is a sigmoid change.

14. The method according to claim **8**, characterized in that said inert change is a change over time.

5

* * * * *

EXHIBIT MM

US006242177B1

(12) **United States Patent**
Simmons et al.

(10) **Patent No.: US 6,242,177 B1**
(45) **Date of Patent: Jun. 5, 2001**

(54) **METHODS AND COMPOSITIONS FOR SECRETION OF HETEROLOGOUS POLYPEPTIDES**

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(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/397,303**

(22) Filed: **Mar. 1, 1995**

(51) **Int. Cl.**[7] **C12Q 1/68**; C07H 21/04; C12P 21/02; C12N 15/67

(52) **U.S. Cl.** **435/6**; 435/69.1; 435/172.3; 536/23.7; 536/24.1

(58) **Field of Search** 435/6, 69.1, 172.3; 536/24.1, 23.7

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,963,495 10/1990 Chang et al. 435/320.1
5,232,840 8/1993 Olins 435/69.1

FOREIGN PATENT DOCUMENTS

177343 4/1986 (EP) .

OTHER PUBLICATIONS

Chang et al., "High–level secretion of human growth hormone by *Escherichia coli*" *Gene* 55:189–196 (1987).

Denefle et al., "Heterologous protein export in *Escherichia coli*: influence of bacterial signal peptides on the export of human interleukin 1β" *Gene* 85:499–510 (1989).

Fujimoto et al., "Expression and secretion of human epidermal growth factor by *Escherichia coli* using enterotoxin signal sequences" *J. Biotech*. 8:77–86 (1988).

Goldstein et al., "Enhancement of Protein Translocation across the Membrane by Specific Mutations in the Hydrophobic Region of the Signal Peptide" *J. Bact.* 172(3):1225–1231 (Mar. 1990).

Gray et al., "Periplasmic production of correctly processed human growth hormone in *Escherichia coli*: natural and bacterial signal sequences are interchangeable" *Gene* 39:247–254 (1985).

Klein et al., "Effects of signal peptide changes on the secretion of bovine somatotropin (bST) from *Escherichia coli*" *Protein Engineering* 5(6):511–517 91992).

Lehnhardt et al., "The Differential Effect on Two Hybrid Proteins of Deletion Mutations within the Hydrophobic Region of the *Escherichia coli* OmpA Signal Peptide" *Journal of Biological Chemistry* 262(4):1716–1719 (Feb. 5, 1987).

Matteucci et al., "Alkaline Phosphatase Fusions: A Tag to Identify Mutations that Result in Increased Expression of Secreted Human Growth Hormone from *E. Coli*" *Biotechnology* 4:51–55 (Jan. 1986).

Morioka–Fujimoto et al., "Modified Enterotoxin Signal Sequences Increase Secretion Level of the Recombinant Human Epidermal Growth Factor in *Escherichia coli* " *Journal of Biological Chemistry* 266(3) :1728–1732 (1991).

Perez–Perez et al., "Increasing the Efficiency of Protein Export in *Escherichia coli*" *Bio/Technology* 12:178–180 (Feb. 12, 1994).

van Dijl et al., "Signal peptidase I overproduction results in increased efficiencies of export and maturation of hybrid secretory proteins in *Escherichia coli*" *Mol. Gen. Genet*. 227:40–48 (1991).

Watson, Marion E. E., "Compilation of published signal sequences" *Nucl. Acids Res.* 12(13):5145–5164 (1984).

Wong et al., "Expression of secreted insulin–like growth factor–1 in *Escherichia coli* " *Gene* 68:193–203 (1988).

Greenberg et al., "High–Level Expression and Secretion of a Lysine–Containing Analog of *Escherichia coli* HeatStable Enterotoxin" *Protein Expression Purification* 2(5–6):394–401 (Oct.–Dec. 1991).

Ibrahimi et al., "A functional interaction between the signal peptide and the translation apparatus is detected by the use of a single point mutation which blocks translation apparatus in dected by . . . " *Journal of Biological Chemistry* 262(21):10189–10194 (Jul. 25, 1987).

Primary Examiner—Nancy Degen
(74) *Attorney, Agent, or Firm*—Janet Hasak; Flehr Hohbach Test Albritton & Herbert LLP

(57) **ABSTRACT**

The instant invention discloses the unexpected result that mutant signal sequences with reduced translational strength provided essentially complete processing and high levels of expression of a polypeptide of interest as compared to wild type signal sequences, and that many mammalian polypeptides require a narrow range of translation levels to achieve maximum secretion. A set of signal sequence vectors provides a range of translational strengths for optimizing expression of a polypeptide of interest.

4 Claims, 21 Drawing Sheets

```
     ecoRI
  1  GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA GTTGTTATTT AAGCTTGCCC AAAAAGAAGA AGAGTCGAAT
     CTTAAGTTGA AGAGGTATGA AACCTATTCC TTTATGTCTG TACTTTTTAG AGTAACGACT CAACAATAAA TTCGAACGGG TTTTTCTTCT TCTCAGCTTA

101  GAACTGTGTG CGCAGGTAGA AGCTTTGGAG ATTATCGTCA CTGCAATGCT TCGCAATATG GCGCAAAATG ACCAACAGCG GTTGATTGAT CAGGTAGAGG
     CTTGACACAC GCGTCCATCT TCGAAACCTC TAATAGCAGT GACGTTACGA AGCGTTATAC CGCGTTTTAC TGGTTGTCGC CAACTAACTA GTCCATCTCC

201  GGGCGCTGTA CGAGGTAAAG CCCGATGCCA GCATTCCTGA CGACGATACG GAGCTGCTGC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTCAGTA
     CCCGCGACAT GCTCCATTTC GGGCTACGGT CGTAAGGACT GCTGCTATGC CTCGACGACG CGCTAATGCA TTTCTTCAAT AACTTCGTAG GAGCAGTCAT

301  AAAAGTTAAT CTTTTCAACA GCTGTCATAA AGTTGTCACG GCCGAGACTT ATAGTCGCTT TGTTTTTATT TTTTAATGTA TTTGTAACTA GTACGCAAGT
     TTTTCAATTA GAAAAGTTGT CGACAGTATT TCAACAGTGC CGGCTCTGAA TATCAGCGAA ACAAAAATAA AAAATTACAT AAACATTGAT CATGCGTTCA

               Trp SD    xbaI        STII SD
401  TCACGTAAAA AGGGTATCTA GAGGTTGAGG TGATTTT    ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT
     AGTGCATTTT TCCCATAGAT CTCCAACTCC ACTAAAA    TAC TTT TTC TTA TAG CGT AAA GAA GAA CGT AGA TAC AAG CAA AAA AGA
  1                                              Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe Ser

486  ATT GCT ACA AAT GCC TAT GCA (SEQ ID NO: 13)
     TAA CGA TGT TTA CGG ATA CGT
 17  Ile Ala Thr Asn Ala Tyr Ala (SEQ ID NO: 14)
```

FIG. 1

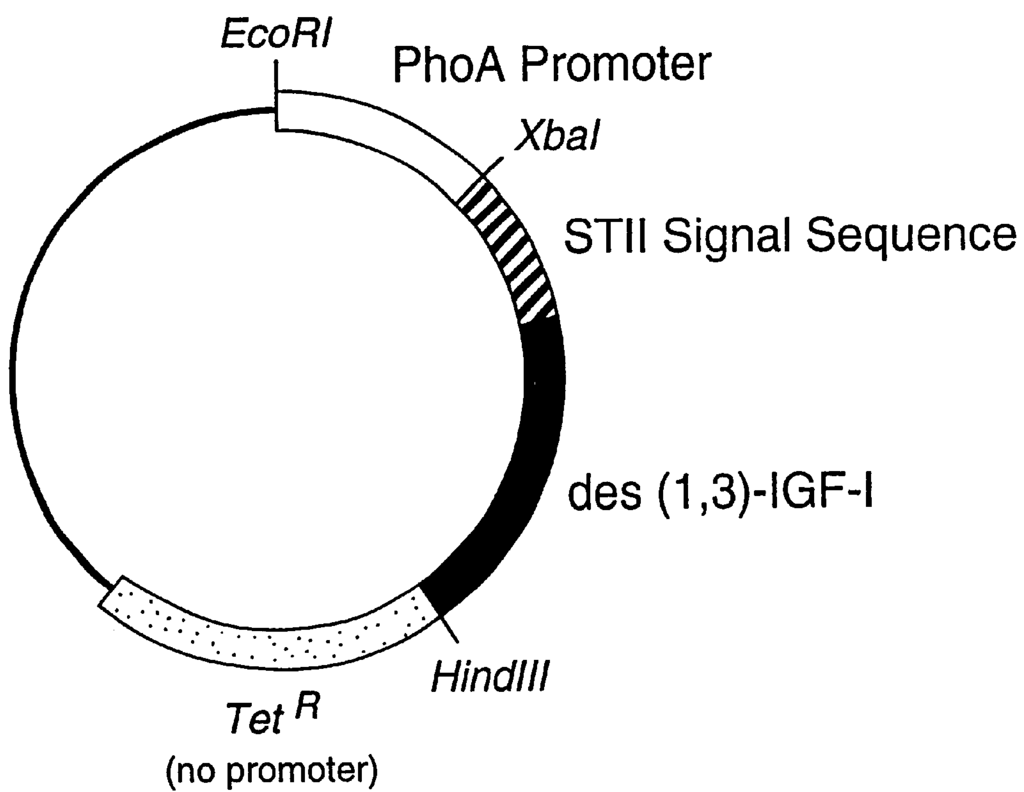

FIG. 2

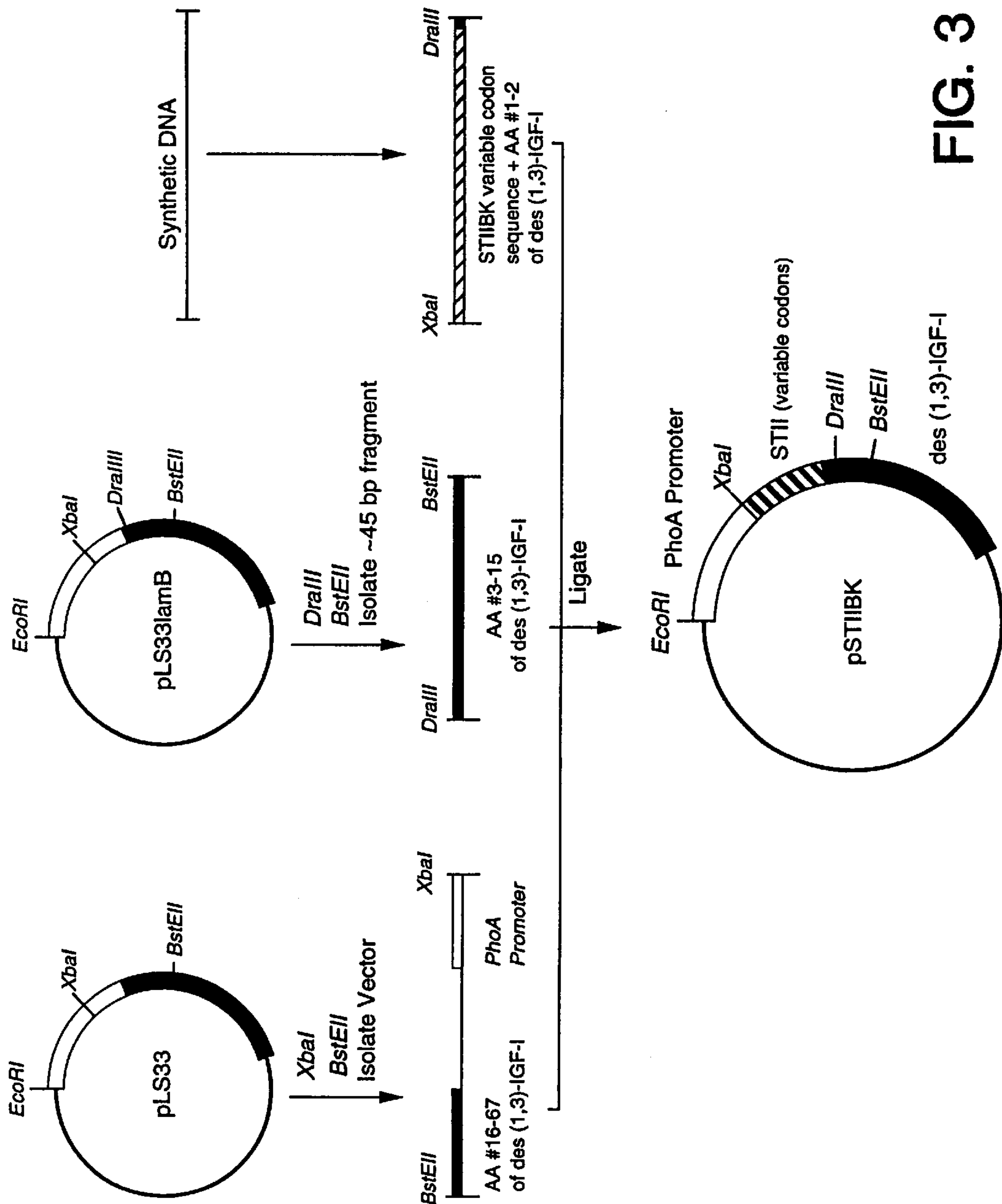

FIG. 3

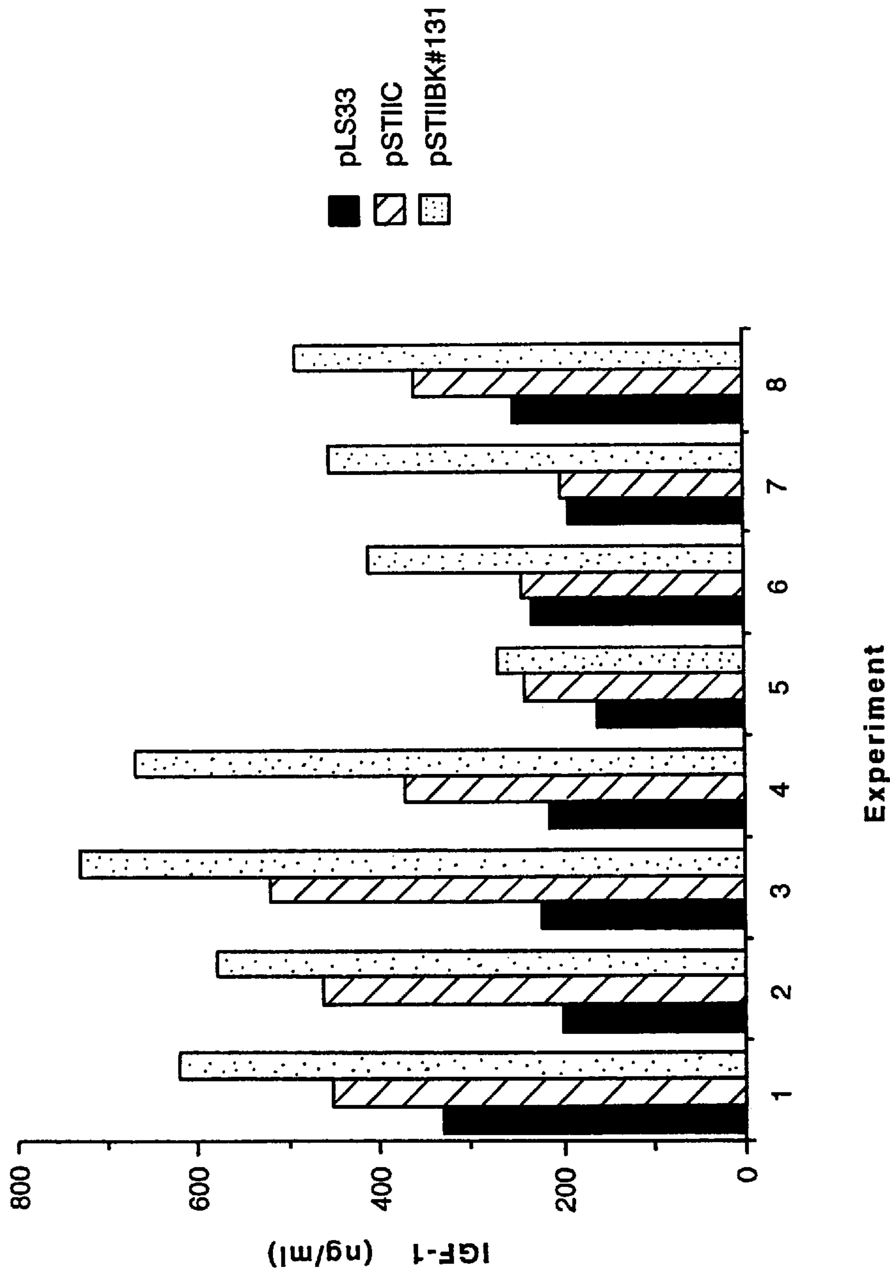

FIG. 4

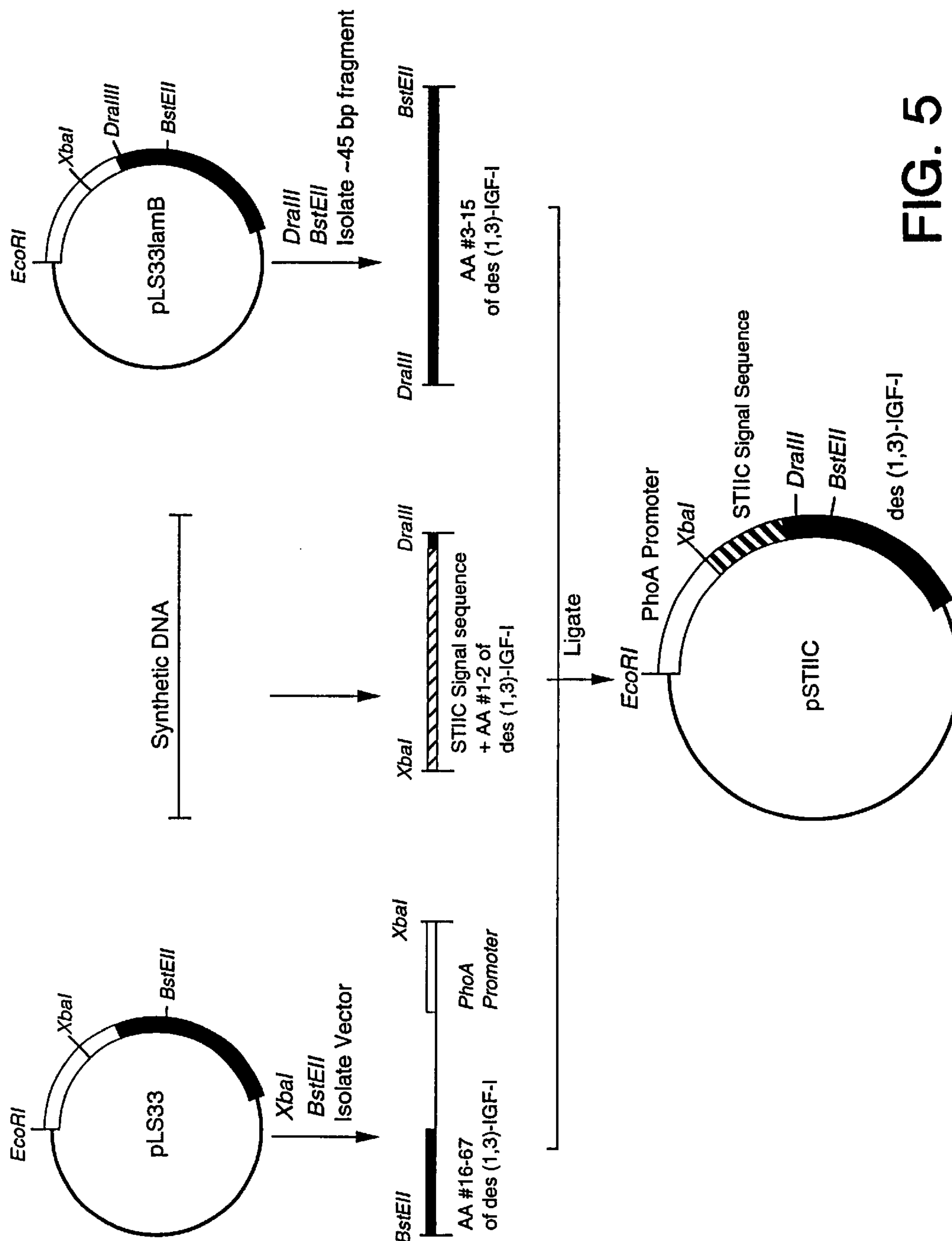

FIG. 5

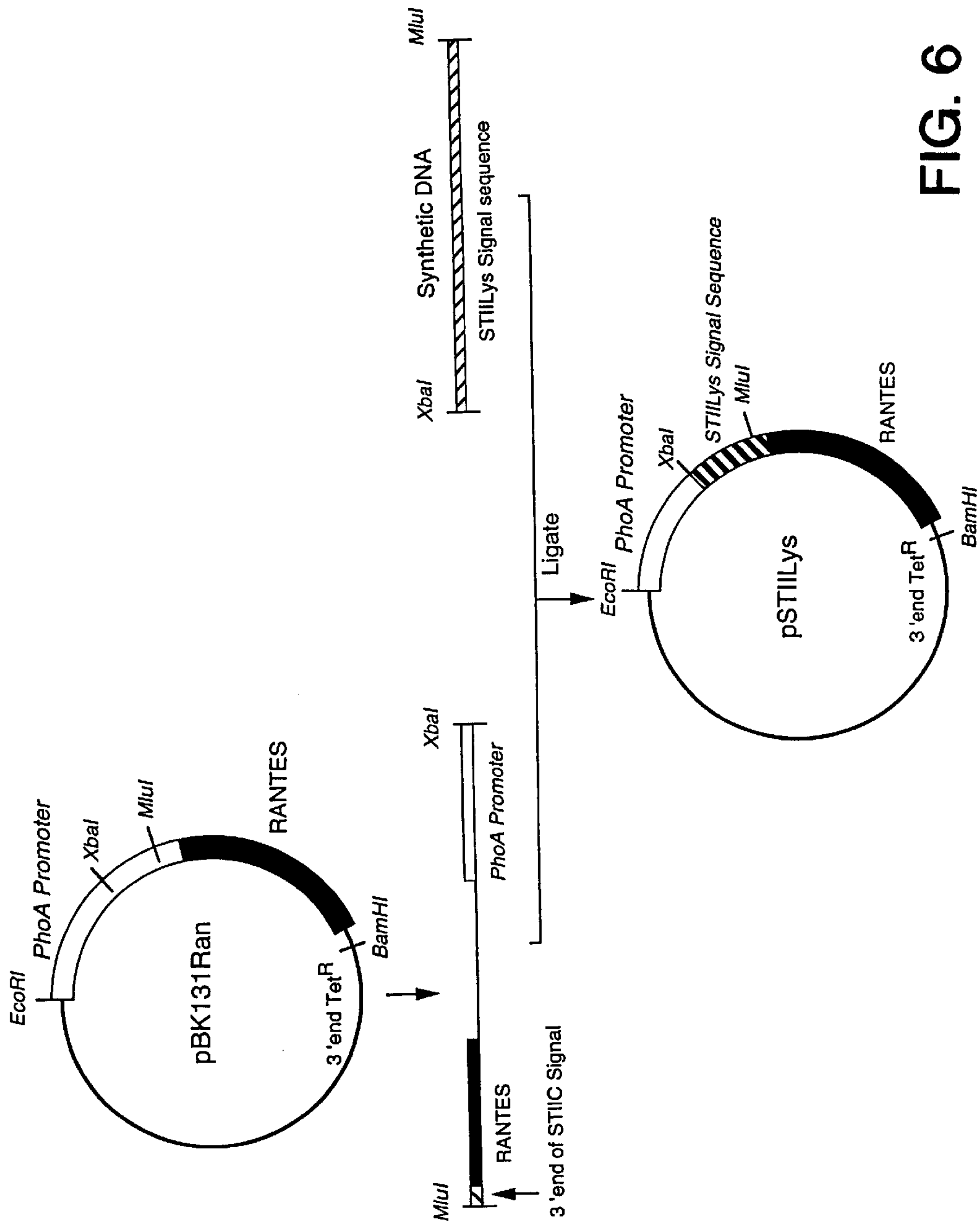

FIG. 6

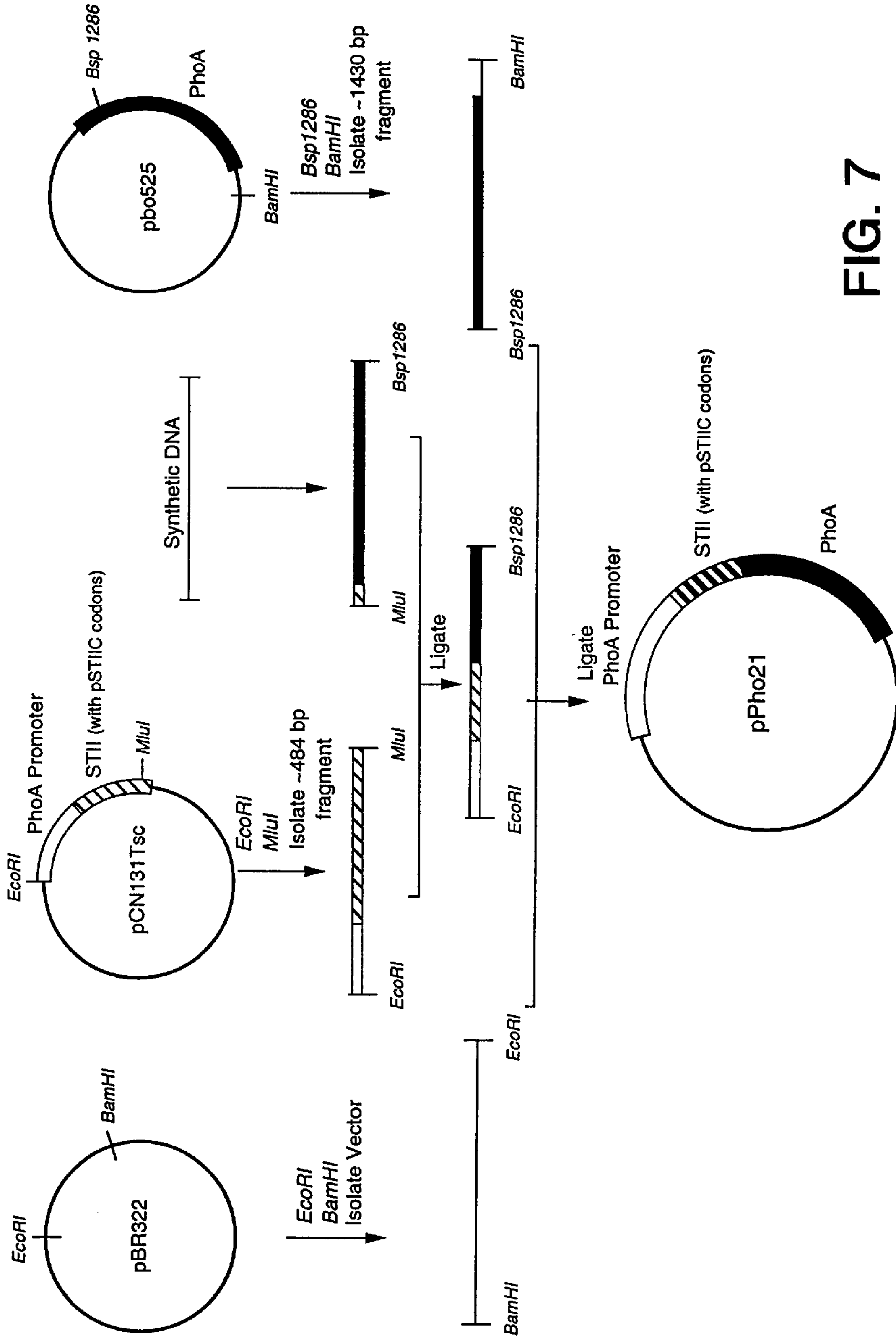

FIG. 7

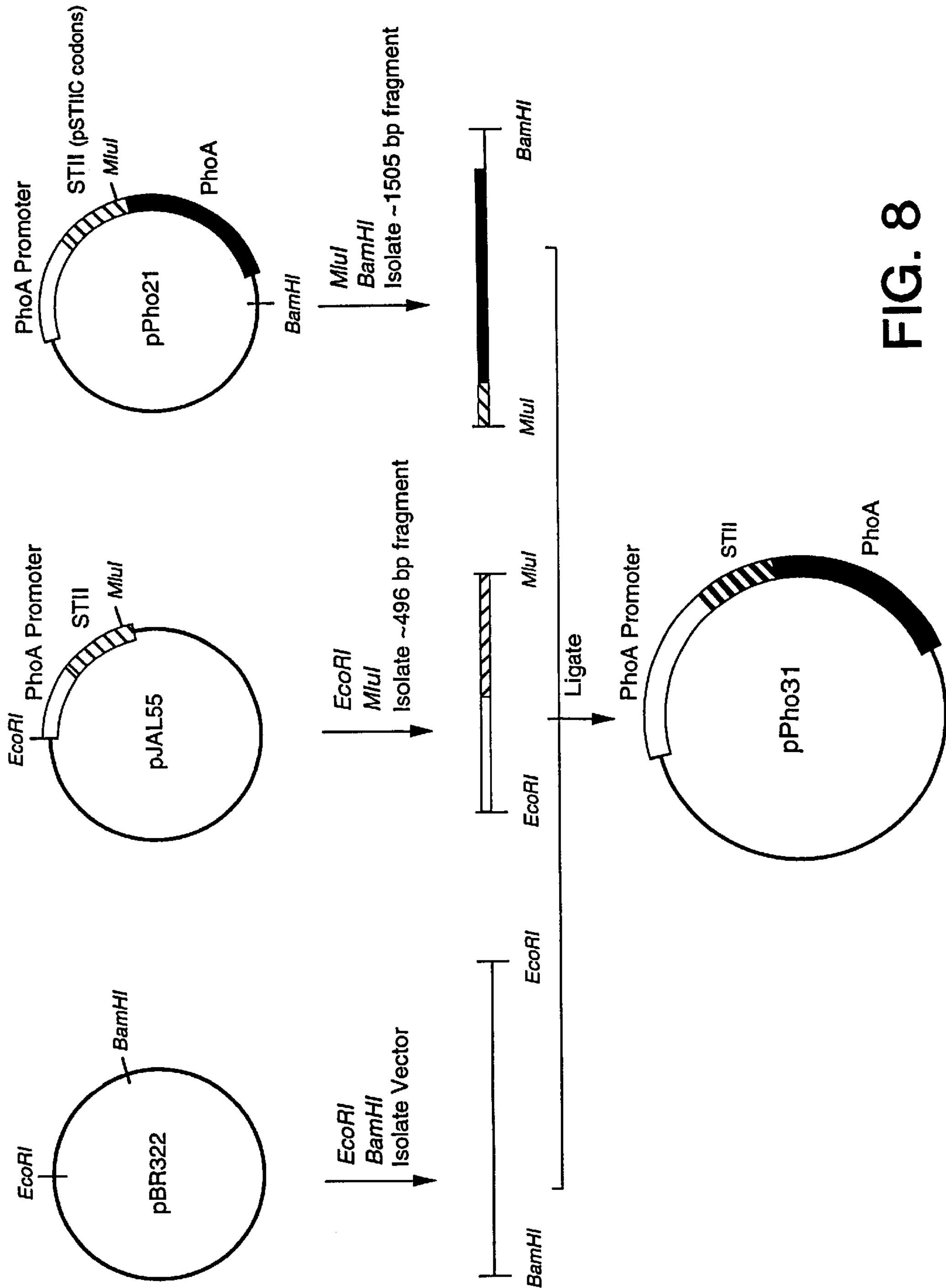

FIG. 8

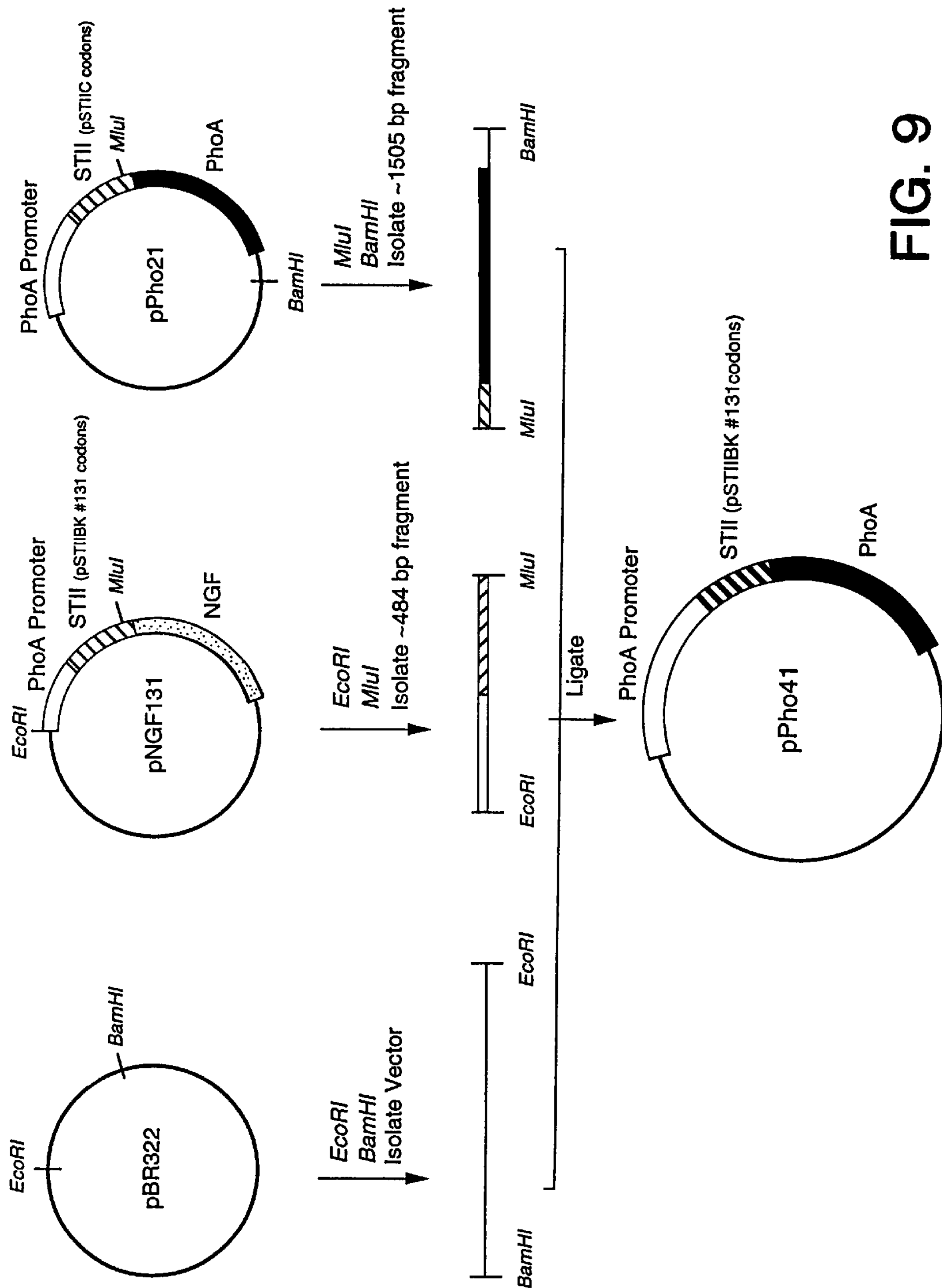

FIG. 9

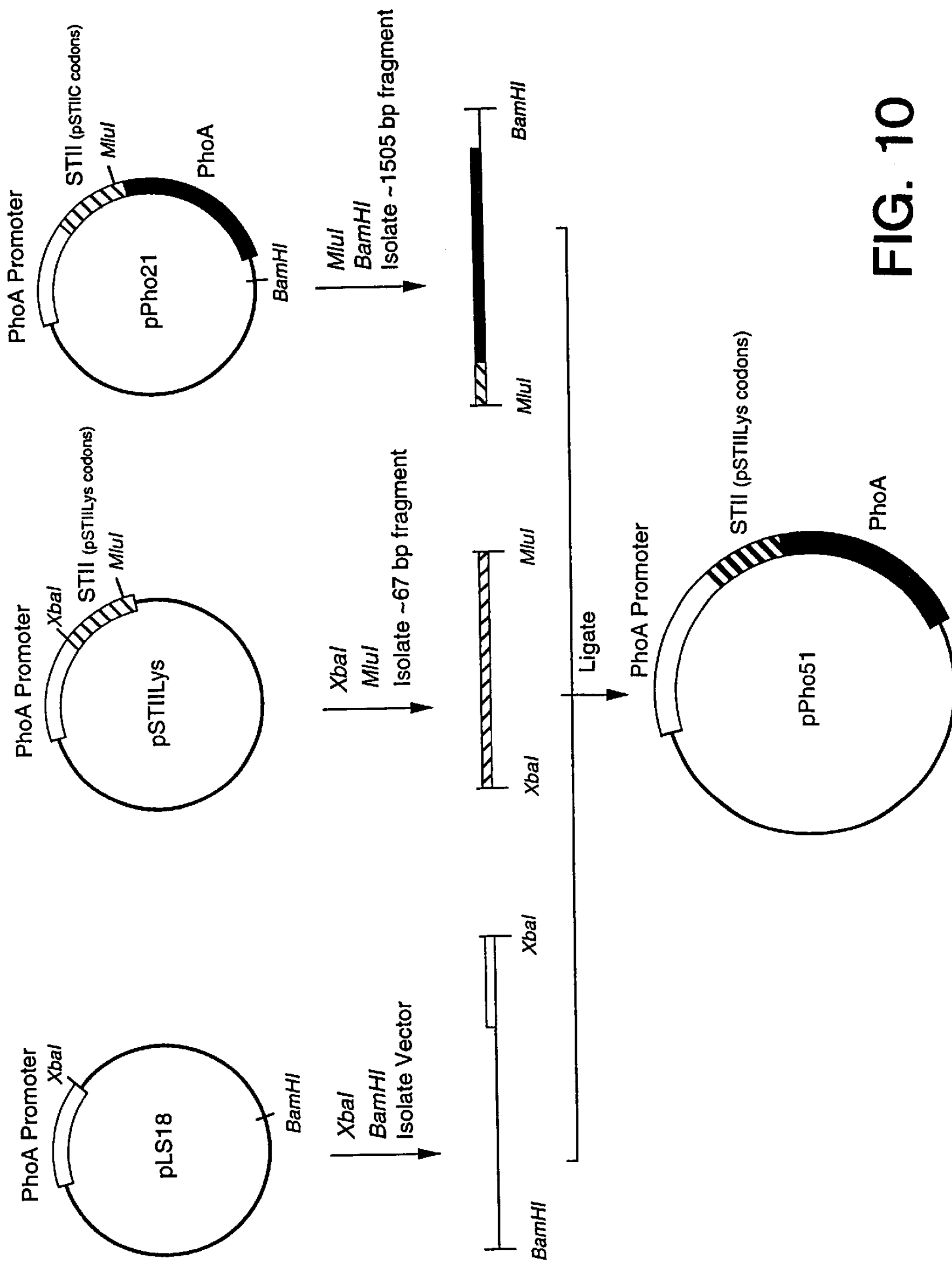

FIG. 10

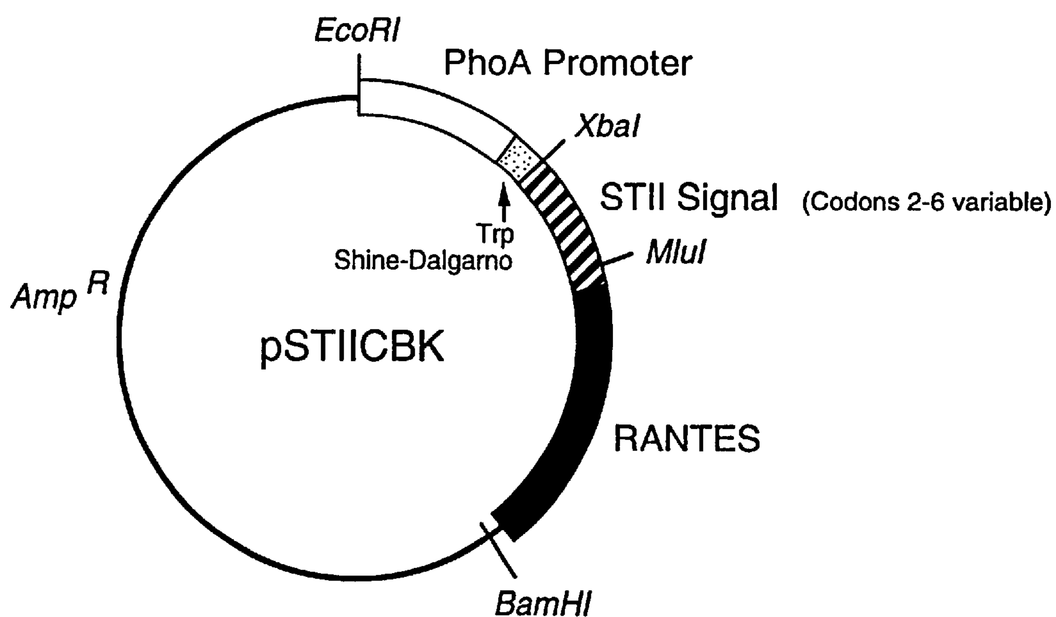

FIG. 11

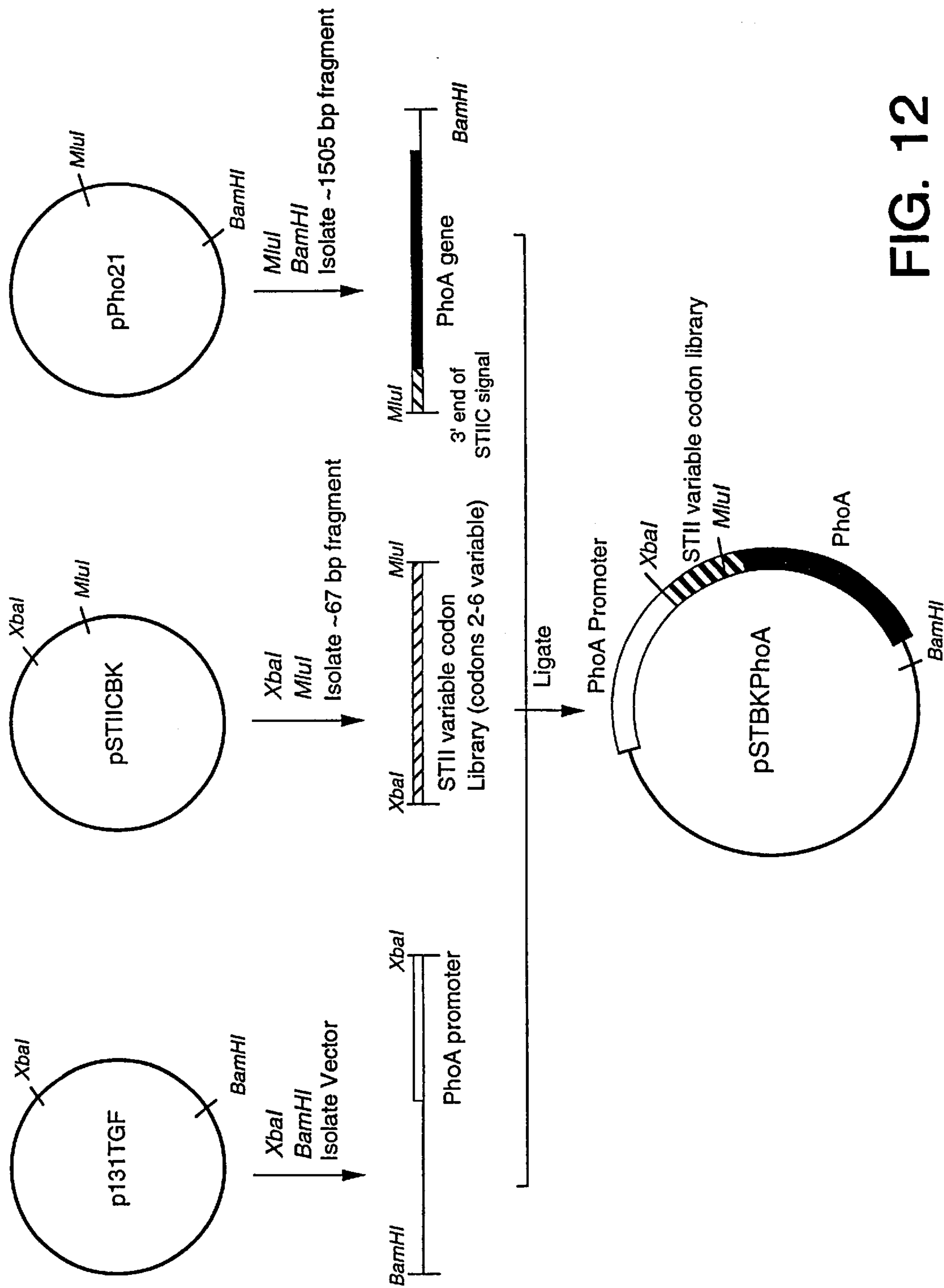

FIG. 12

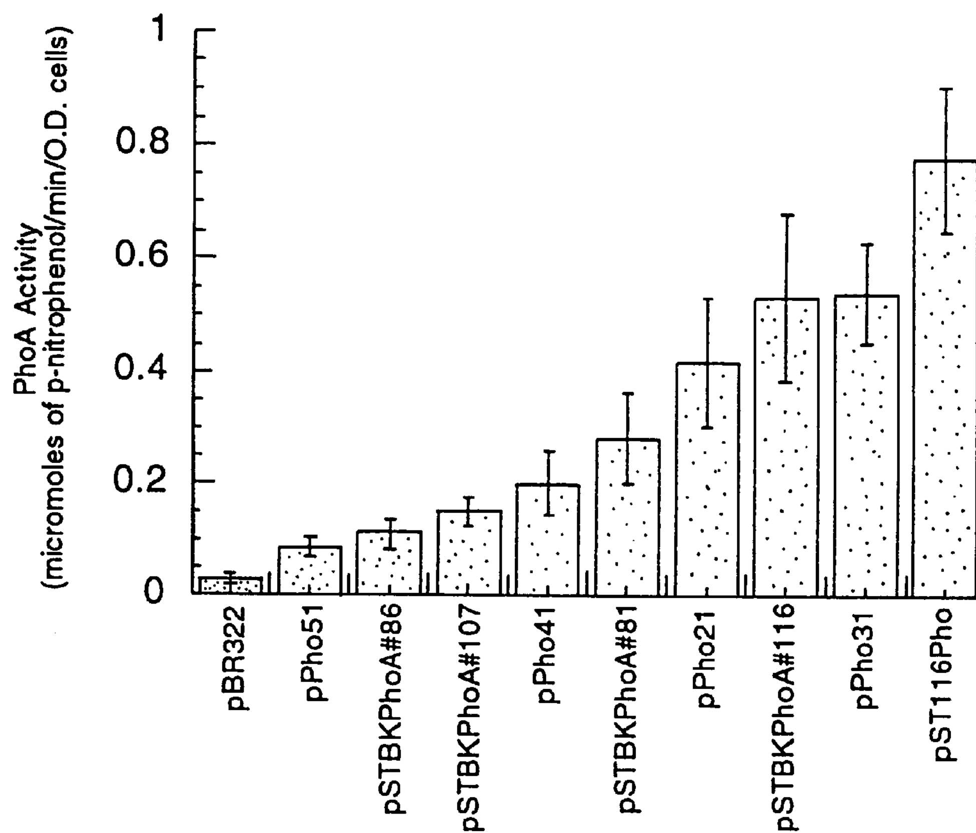

FIG. 13

pPho31 (Wild type STII + MluI site)
TCTAGAGGTTGAGGTGATTTT ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT

pPho21 (STIIC)
TCTAGAATT ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT

pPho41 (STIIBK#131)
TCTAGAATT ATG AAG AAG AAT ATT GCG TTC CTA CTT GCC TCT ATG TTT GTC

pPho51 (STIILys - unless otherwise noted this sequence is the TIR=1 used in the examples)
TCTAGAATT ATG AAG AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT

pSTBKPhoA#116
TCTAGAATT ATG AAA AAA AAC ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT

pSTBKPhoA#81
TCTAGAATT ATG AAA AAA AAC ATT GCC TTT CTT CTT GCA TCT ATG TTC GTT

pSTBKPhoA#107
TCTAGAATT ATG AAG AAA AAC ATC GCT TTT CTT CTT GCA TCT ATG TTC GTT

pSTBKPhoA#86
TCTAGAATT ATG AAA AAG AAC ATA GCG TTT CTT CTT GCA TCT ATG TTC GTT

pST116Pho
TCTAGAGGTTGAGGTGATTTT ATG AAA AAA AAC ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT

FIG. 14A

		TIR RELATIVE STRENGTH
TTT TCT ATT GCT ACA AAY GCS TAT GCM*	(SEQ ID NO:15)	9
TTT TCT ATT GCT ACA AAC GCG TAT GCM	(SEQ ID NO:16)	7
TTT TCT ATA GCT ACA AAC GCG TAT GCM	(SEQ ID NO:17)	3
TTT TCT ATT GCT ACA AAC GCG TAT GCM	(SEQ ID NO:18)	1
TTT TCT ATT GCT ACA AAC GCG TAT GCM	(SEQ ID NO:19)	9
TTT TCT ATT GCT ACA AAC GCG TAT GCM	(SEQ ID NO:20)	4
TTT TCT ATT GCT ACA AAC GCG TAT GCM	(SEQ ID NO:21)	2
TTT TCT ATT GCT ACA AAC GCG TAT GCM	(SEQ ID NO:22)	1
TTT TCT ATT GCT ACA AAC GCG TAT GCM	(SEQ ID NO:23)	13

* The codons for the last four amino acids of this sequence may differ in some of the examples of protein secretion. For example, in the IGF-1, VEGF165 and RANTES secretion plasmids, the sequence is AAT GCC TAT GCA.
The last codon for the last amino acid in every sequence listed may vary in the examples of protein secretion - GCC and GCA were both used.

FIG. 14B

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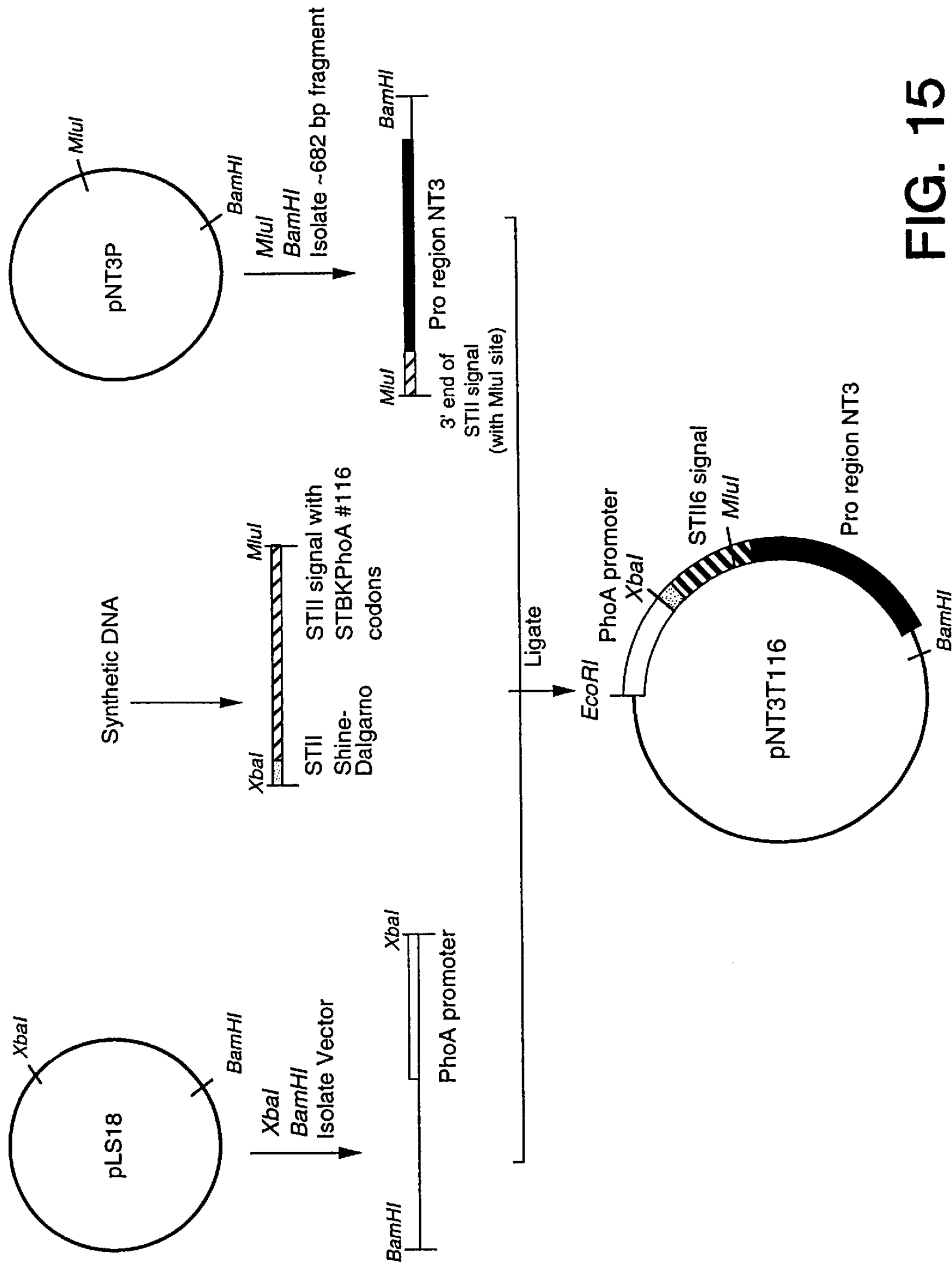


FIG. 15

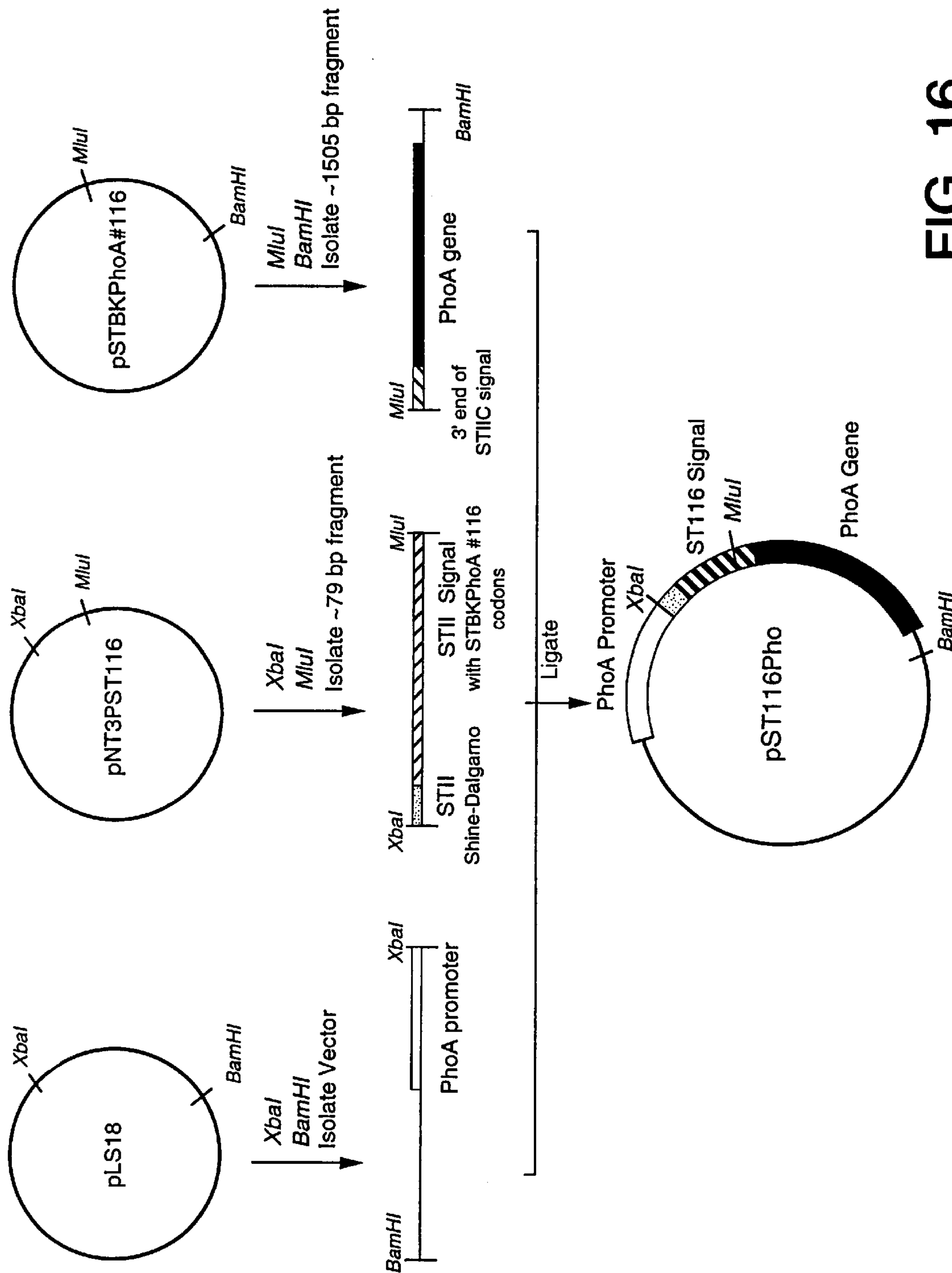
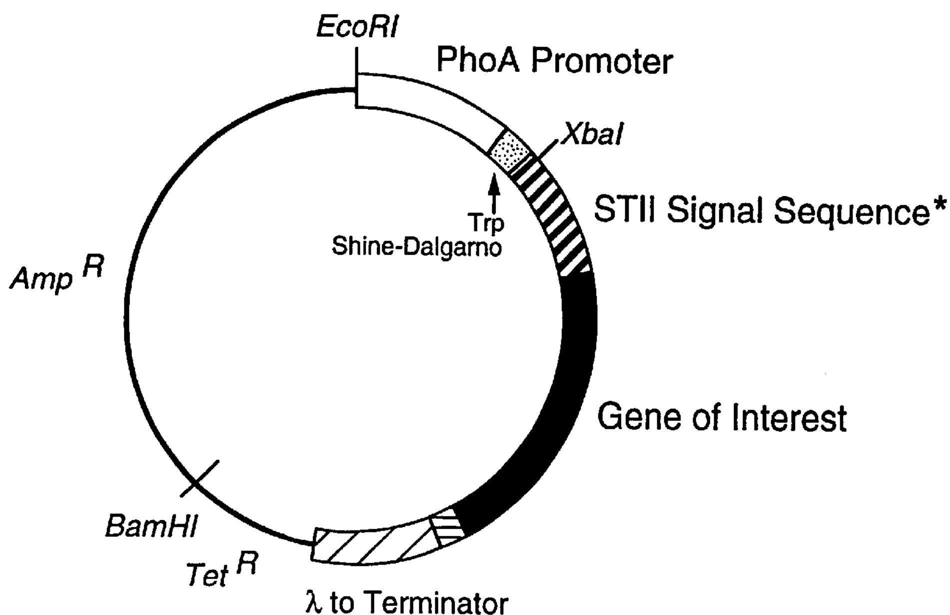
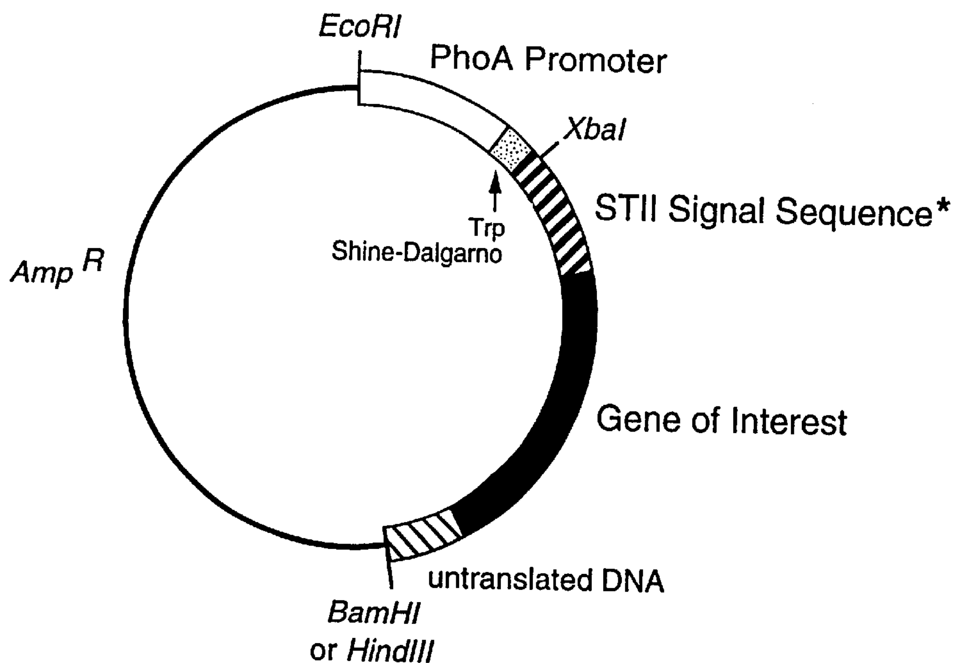


FIG. 16



* One of the nucleotide sequences listed in Figure 14 (STII Shine-Dalgarno may also be included).

FIG. 17



* One of the nucleotide sequences listed in Figure 14 (STII Shine-Dalgarno may also be included).

FIG. 18

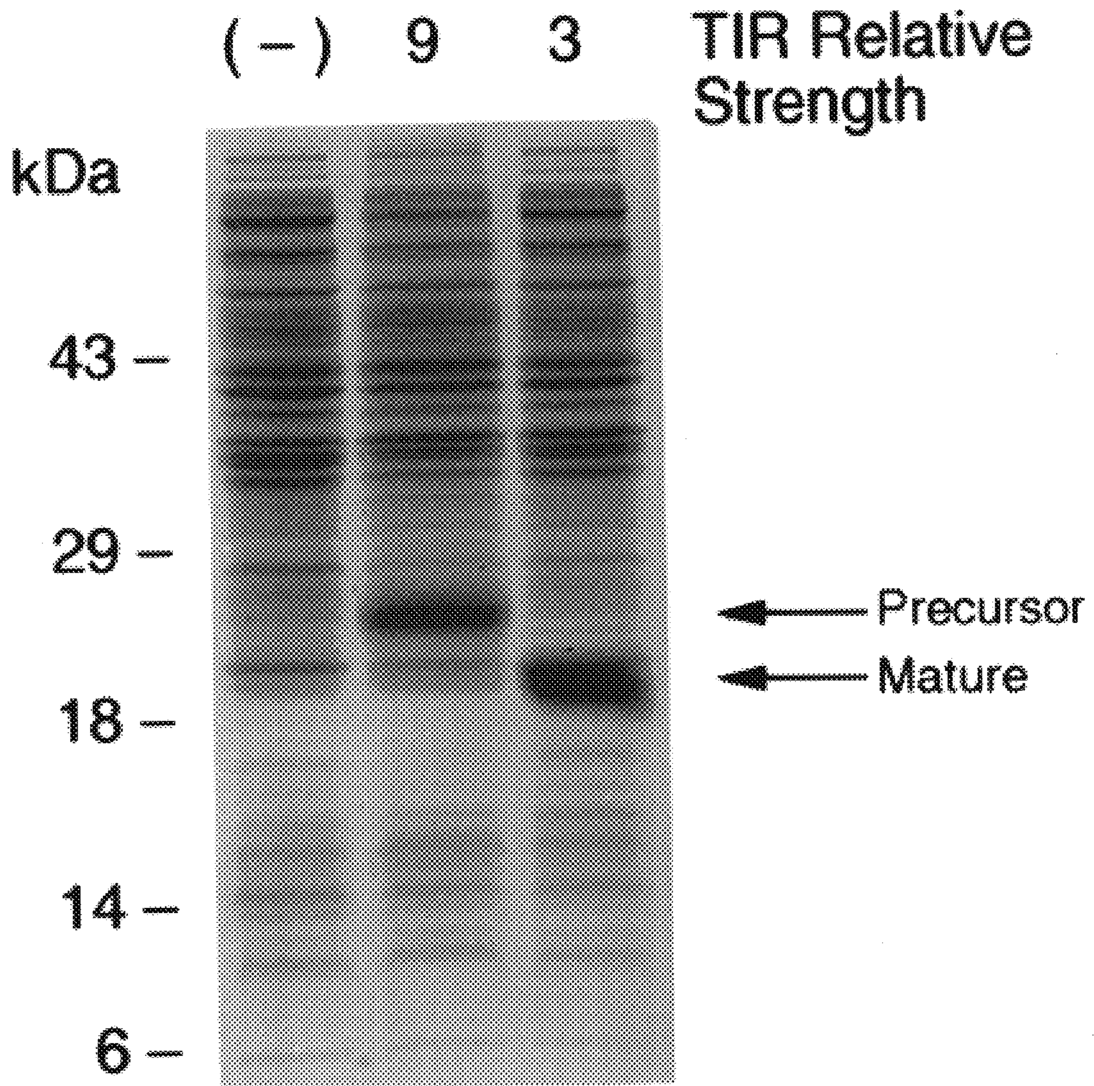


FIG. 19

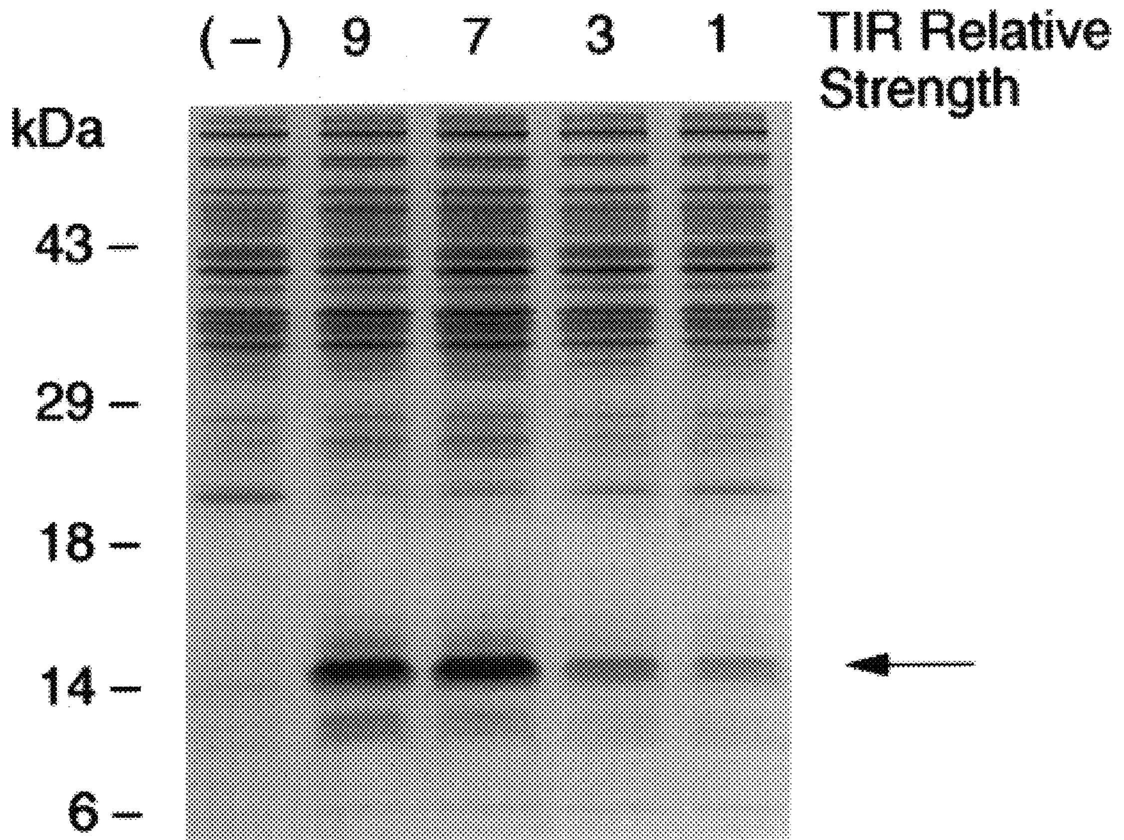


FIG. 20

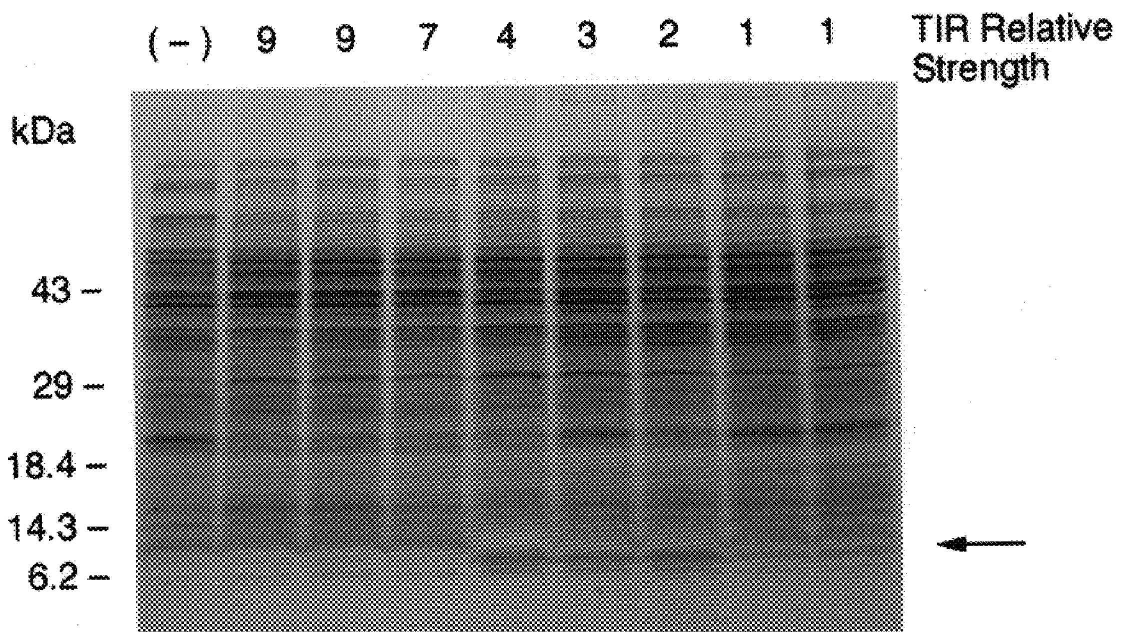


FIG. 21

METHODS AND COMPOSITIONS FOR SECRETION OF HETEROLOGOUS POLYPEPTIDES

FIELD OF THE INVENTION

This invention relates to signal sequences for the secretion of heterologous polypeptides from bacteria.

DESCRIPTION OF BACKGROUND AND RELATED ART

Secretion of heterologous polypeptides into the periplasmic space of *E. coli* and other prokaryotes or into their culture media is subject to a variety of parameters. Typically, vectors for secretion of a polypeptide of interest are engineered to position DNA encoding a secretory signal sequence 5' to the DNA encoding the polypeptide of interest. Two major recurring problems plague the secretion of such polypeptides. First, the signal sequence is often incompletely processed or removed, and second, the amount of polypeptide secreted is often low or undetectable. Attempts to overcome these problems fall into three major areas: trying several different signal sequences, mutating the amino acid sequence of the signal sequence, and altering the secretory pathway within the host bacterium.

A number of signal sequences are available for the first approach in overcoming secretion problems. Watson (*Nucleic Acids Research*12: 5145–5164 (1984)) discloses a compilation of signal sequences. U.S. Pat. No. 4,963,495 discloses the expression and secretion of mature eukaryotic protein in the periplasmic space of a host organism using a prokaryotic secretion signal sequence DNA linked at its 3' end to the 5' end of the DNA encoding the mature protein. In particular, the DNA encoding *E. coli* enterotoxin signals, especially STII, are preferred. Chang et al. (*Gene*55:189–196 (1987)) discloses the use of the STII signal sequence to secrete hGH in *E. coli.* Gray et al. (*Gene*39:247–245 (1985)) disclose the use of the natural signal sequence of human growth hormone and the use of the *E. coli* alkaline phosphatase promoter and signal sequence for the secretion of human growth hormone in *E. coli.* Wong et al. (*Gene*68:193–203 (1988)) disclose the secretion of insulin-like growth factor 1 (IGF-1) fused to LamB and OmpF secretion leader sequences in *E. coli*, and the enhancement of processing efficiency of these signal sequences in the presence of a prlA4 mutation. Fujimoto et al. (*J. Biotech.*8:77–86 (1988)) disclose the use of four different *E. coli* enterotoxin signal sequences, STI, STII, LT-A, and LT-B for the secretion of human epidermal growth factor (hEGF) in *E. coli.* Denefle et al. (*Gene*85: 499–510 (1989)) disclose the use of OmpA and PhoA signal peptides for the secretion of mature human interleukin 1β.

Mutagenesis of the signal sequence has, in general, not been especially helpful in overcoming secretion problems. For example, Morioka-Fujimoto et al. (*J. Biol. Chem.*266:1728–1732 (1991)) disclose amino acid changes in the LTA signal sequence that increased the amount of human epidermal growth factor secreted in *E. coli*. Goldstein et al. (*J. Bact.*172:1225–1231 (1990)) disclose amino acid substitution in the hydrophobic region of OmpA effected secretion of nuclease A but not TEM β-lactamase. Matteucci et al. (*Biotech.*4:51–55 (1986)) disclose mutations in the signal sequence of human growth hormone that enhance secretion of hGH. Lehnhardt et al. (*J. Biol. Chem.*262:1716–1719 (1987) disclose the effect of deletion mutations in OmpA signal peptide on secretion of nuclease A and TEM β-lactamase.

Finally, attempts at improving heterologous secretion in *E. coli* by modulating host machinery has so far shown limited improvement in overcoming secretion problems. For example, van Dijl et al. (*Mol. Gen. Genet.*227:40–48 (1991)) disclose the effects of overproduction of the *E. coli* signal peptidase I (SPase I) on the processing of precursors. Klein et al. (*Protein Engineering*5:511–517 (1992) disclose that mutagenesis of the LamB signal sequence had little effect on secretion of bovine somatotropin, and that secretion properties of bovine somatotropin appear to be determined by the mature protein rather than by changes in the signal sequence. Perez-Perez et al. (*Bio/Technology*12:179–180 (1994)) disclose that providing an *E. coli* host with additional copies of prlA4 (secY allele) and secE genes, which encode the major components of the "translocator", i.e., the molecular apparatus that physically moves proteins across the membrane, increased the ratio of mature to precursor hIL-6 from 1.2 to 10.8. U.S. Pat. No. 5,232,840 discloses novel ribosome binding sites useful in enhancing protein production in bacteria through enhanced and/or more efficient translation. U.S. Pat. No. 5,082,783 discloses improved secretion of heterologous proteins by hosts such as yeasts by using promoters of at most intermediate strength with heterologous DNA secretion signal sequences. European Patent Application No. 84308928.5, filed Dec. 19, 1984, discloses promoter-ribosome binding site expression elements of general utility for high level heterologous gene expression.

The instant invention discloses the unexpected result that altered translation initiation regions with reduced translational strength provided essentially complete processing and high levels of secretion of a polypeptide of interest as compared to wild type sequences, and that many mammalian polypeptides require a narrow range of translation levels to achieve maximum secretion. A set of vectors with variant translation initiation regions provides a range of translational strengths for optimizing secretion of a polypeptide of interest.

SUMMARY OF THE INVENTION

One aspect of the invention is a method of optimizing secretion of a heterologous polypeptide of interest in a cell comprising comparing the levels of expression of the polypeptide under control of a set of nucleic acid variants of a translation initiation region, wherein the set of variants represents a range of translational strengths, and determining the optimal translational strength for production of mature polypeptide, wherein the optimal translational strength is less than the translational strength of the wild-type translation initiation region.

In a further aspect of the invention the variants are signal sequence variants, especially variants of the STII signal sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts the sequence of the PhoA promoter, Trp and STII Shine-Dalgarno regions and STII signal sequence.

FIG. **2** is a diagram depicting relevant features of the plasmid pLS33.

FIG. **3** is a diagram depicting construction of the library, pSTIIBK.

FIG. **4** is a graph depicting comparison of the levels of expression of IGF-1, as measured by the amount of IGF-1 detected in culture supernatants, for pLS33, pSTIIBK#131, and pSTIIC. Experiments 1 to 8 represent measurements taken on 8 separate dates.

FIG. **5** is a diagram depicting construction of the plasmid pSTIIC.

FIG. **6** is a diagram depicting construction of the plasmid pSTIILys.

FIG. **7** is a diagram depicting construction of the plasmid pPho21.

FIG. **8** is a diagram depicting construction of the plasmid pPho31.

FIG. **9** is a diagram depicting construction of the plasmid pPho41.

FIG. **10** is a diagram depicting construction of the plasmid pPho51.

FIG. **11** is a diagram depicting relevant features of the library, pSTIICBK.

FIG. **12** is a diagram depicting construction of the library, pSTBKPhoA.

FIG. **13** is a graph depicting PhoA activity in isolates of the pSTBKPhoA library.

FIG. **14** depicts the nucleotide sequences of the listed STII signal sequence variants.

FIG. **15** is a diagram depicting construction of the plasmid pNT3PST116.

FIG. **16** is a diagram depicting construction of the plasmid pST116Pho.

FIG. **17** is a diagram depicting relevant features of "category A" plasmids used in the examples.

FIG. **18** is a diagram depicting relevant features of "category B" plasmids used in the examples.

FIG. **19** is a photograph of a Coomassie blue stained polypeptide gel depicting secretion of mature ICAM-1 in *E. coli* under control of variant STII signal sequences. The TIR of relative strength 9 was provided by the pPho3 STII variant; the TIR of relative strength 3 was provided by the pPho41 STII variant. Precursor and mature forms of the polypeptide are indicated in the figure.

FIG. **20** is a photograph of a Coomassie blue stained polypeptide gel depicting secretion of mature NT3 in *E. coli* under control of variant STII signal sequences. The TIR of relative strength 9 was provided by the pPho31 STII variant; the TIR of relative strength 7 was provided by the pPho21 STII variant; the TIR of relative strength 3 was provided by the pPho41 STII variant; the TIR of relative strength 1 was provided by the pPho51 STII variant. The mature form of the polypeptide is indicated in the figure.

FIG. **21** is a photograph of a Coomassie blue stained polypeptide gel depicting secretion of mature RANTES in *E. coli* under control of variant STII signal sequences. Reading from left to right in the figure, the TIRs of relative strength 9 were provided by the pPho31and the pSTBK-PhoA#116 STII variants; the TIR of relative strength 7 was provided by the pPho21 STII variant; the TIR of relative strength 4 was provided by the pSTBKPhoA#81 STII variant; the TIR of relative strength 3 was provided by the pPho41 STII variant, the TIR of relative strength 2 was provided by the pSTBKPhoA#107 STII variant; the TIRs of relative strength 1 were provided by the pSTBKPhoA#86 and the pPho51 STII variants. The mature form of the polypeptide is indicated in the figure.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A. Definitions

The "translation initiation region" or TIR, as used herein refers to a region of RNA (or its coding DNA) determining the site and efficiency of initiation of translation of a gene of interest. (See, for example, McCarthy et al. *Trends in Genetics*6:78–85 (1990).). A TIR for a particular gene can extend beyond the ribosome binding site (rbs) to include sequences 5' and 3' to the rbs. The rbs is defined to include, minimally, the Shine-Dalgarno region and the start codon, plus the bases in between, but can include the expanse of mRNA protected from ribonuclease digestion by bound ribosomes. Thus, a TIR can include an untranslated leader or the end of an upstream cistron, and thus a translational stop codon.

A "secretion signal sequence" or "signal sequence" as used herein refers to a sequence present at the amino terminus of a polypeptide that directs its translocation across a membrane. Typically, a precursor polypeptide is processed by cleavage of the signal sequence to generate mature polypeptide.

The term "translational strength" as used herein refers to a measurement of a secreted polypeptide in a control system wherein one or more variants of a TIR is used to direct secretion of a polypeptide encoded by a reporter gene and the results compared to the wild-type TIR or some other control under the same culture and assay conditions. For example, in these experiments translational strength is measured by using alkaline phosphatase as the reporter gene expressed under basal level control of the PhoA promoter, wherein secretion of the PhoA polypeptide is directed by variants of the STII signal sequence. The amount of mature alkaline phosphatase present in the host is a measure of the amount of polypeptide secreted, and can be quantitated relative to a negative control. Without being limited to any one theory, "translational strength" as used herein can thus include, for example, a measure of mRNA stability, efficiency of ribosome binding to the ribosome binding site, and mode of translocation across a membrane.

"Polypeptide" as used herein refers generally to peptides and polypeptides having at least about two amino acids.

B. General Methods

The instant invention demonstrates that translational strength is a critical factor in determining whether many heterologous polypeptides are secreted in significant quantities. Thus, for a given TIR, a series of amino acid or nucleic acid sequence variants can be created with a range of translational strengths, thereby providing a convenient means by which to adjust this factor for the optimal secretion of many different polypeptides. The use of a reporter gene expressed under the control of these variants, such as PhoA, provides a method to quantitate the relative translational strengths of different translation initiation regions. The variant or mutant TIRs can be provided in the background of a plasmid vector thereby providing a set of plasmids into which a gene of interest may be inserted and its expression measured, so as to establish an optimum range of translational strengths for maximal expression of mature polypeptide.

Thus, for example, signal sequences from any prokaryotic or eukaryotic organism may be used. Preferably, the signal sequence is STII, OmpA, PhoE, LamB, MBP, or PhoA.

Mutagenesis of the TIR is done by conventional techniques that result in codon changes which can alter the amino acid sequence, although silent changes in the nucleotide sequence are preferred. Alterations in the TIR can include, for example, alterations in the number or spacing of Shine-Dalgarno sequences, along with alterations in the signal sequence. One preferred method for generating mutant signal sequences is the generation of a "codon bank" at the beginning of a coding sequence that does not change

the amino acid sequence of the signal sequence (i.e., the changes are silent). This can be accomplished by changing the third nucleotide position of each codon; additionally, some amino acids, such as leucine, serine, and arginine, have multiple first and second positions that can add complexity in making the bank. This method of mutagenesis is described in detail in Yansura et al. (*METHODS: A Companion to Methods in Enzymol.*4:151–158 (1992)). Basically, a DNA fragment encoding the signal sequence and the beginning of the mature polypeptide is synthesized such that the third (and, possibly, the first and second, as described above) position of each of the first 6 to 12 codons is altered. The additional nucleotides downstream of these codons provide a site for the binding of a complementary primer used in making the bottom strand. Treatment of the top coding strand and the bottom strand primer with DNA polymerase I (Klenow) will result in a set of duplex DNA fragments containing randomized codons. The primers are designed to contain useful cloning sites that can then be used to insert the DNA fragments in an appropriate vector, thereby allowing amplification of the codon bank. Alternative methods include, for example, replacement of the entire rbs with random nucleotides (Wilson et al., *BioTechniques*17:944–952 (1994)), and the use of phage display libraries (see, for example, Barbas et al., *Proc. Natl. Acad. Sci. U.S.A.*89:4457–4461 (1992); Garrard et al., *Gene*128:103–109 (1993)).

Typically, the TIR variants will be provided in a plasmid vector with appropriate elements for expression of a gene of interest. For example, a typical construct will contain a promoter 5' to the signal sequence, a restriction enzyme recognition site 3' to the signal sequence for insertion of a gene of interest or a reporter gene, and a selectable marker, such as a drug resistance marker, for selection and/or maintenance of bacteria transformed with the resulting plasmids.

Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems (Chang et al., *Nature*275:617–624 (1978); and Goeddel et al., *Nature*281:544–548 (1979)), alkaline phosphatase, a tryptophan (Trp) promoter system (Goeddel, *Nucleic Acids Res.*8(18):4057–4074 (1980) and EP 36,776) and hybrid promoters such as the tac promoter (deBoer et al., *Proc. Natl. Acad. Sci. U.S.A.*80:2114 25 (1983).

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.*255(24):12073–80 (1980)) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.*7:149–67 (1968)); and Holland, *Biochemistry*17:4900–4907 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phospho-fructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman et al., EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

Any reporter gene may be used which can be quantified in some manner. Thus, for example, alkaline phosphatase production can be quantitated as a measure of the secreted level of the phoA gene product. Other examples include, for example, the β-lactamase genes.

Preferably, a set of vectors is generated with a range of translational strengths into which DNA encoding a polypeptide of interest may be inserted. This limited set provides a comparison of secreted levels of polypeptides. The secreted level of polypeptides can be determined, for example, by a functional assays for the polypeptide of interest, if available, radioimmunoassays (RIA), enzyme-linked immunoassays (ELISA), or by PAGE and visualization of the correct molecular weight of the polypeptide of interest. Vectors so constructed can be used to transform an appropriate host. Preferably, the host is a prokaryotic host. More preferably, the host is *E. coli.*

Further details of the invention can be found in the following examples, which further define the scope of the invention. All references cited herein are expressly incorporated by reference in their entirety.

EXAMPLES

I. Plasmid Constucts

A. Basic Plasmid Construction

All of the plasmids described in this patent application were constructed from a basic backbone of pBR322 (Sutcliffe, *Cold Spring Harb Symp Quant Biol.*43:77–90 (1978)). While the gene of interest expressed in each case varies, the transcriptional and translational sequences required for the expression of each gene were provided by the PhoA promoter and the Trp Shine-Dalgarno sequence (Chang et al., *Gene*55:189–196 (1987)). Additionally, in the cases noted, a second Shine-Dalgarno sequence, the STII Shine-Dalgarno sequence (Picken et al., *Infect Immun*42(1):269–275 (1983)), was also be present. Secretion of the polypeptide was directed by the STII signal sequence or variants thereof (Picken et al., *Infect Immun*42 (1):269–275 (1983)). The PhoA promoter, Trp and STII Shine-Dalgarno sequences and the sequence of the wild-type STII signal sequence are given in FIG. **1**.

B. Construction of pLS33

The plasmid pLS33 was derived from phGH1 (Chang et al., *Gene*55:189–196 (1987)), which was constructed for the expression of des(1,3)-IGF-I. In the plasmid pLS33, the gene encoding this version of insulin-like growth factor I (altered from the original sequence (Elmblad et al., *Third European Congress on Biotechnology III*. Weinheim: Verlag Chemie, pp.287–292 (1984)) by the removal of the first three amino acids at the N-terminus) replaced the gene encoding human growth hormone. The construction pLS33 maintained the sequences for the PhoA promoter, Trp and STII Shine-Dalgarno regions and the wild-type STII signal sequence described for phGH1. However, the 3' end following the termination codon for des(1, 3)-IGF-I was altered from that described for phGH1. In the case of pLS33, immediately downstream of the termination codon a HindIII restriction site was engineered, followed by the methionine start codon of the tetracycline resistance gene of pBR322 (Sutcliffe, *Cold Spring Harb Symp Quant Biol*43:77–90 (1978)). A diagram of the plasmid pLS33 is given in FIG. **2**.

C. Construction of pSTIIBK

A plasmid library containing a variable codon bank of the STII signal sequence (pSTIIBK) was constructed to screen for improved nucleotide sequences of this signal. The vector fragment for the construction of pSTIIBK was created by isolating the largest fragment when pLS33 was digested with XbaI and BstEII. This vector fragment contains the sequences that encode the PhoA promoter, Trp Shine-Dalgarno sequence and amino acids 16–67 of des(1,3)-IGF-

I. The coding region for amino acids 3–15 of des(1,3)-IGF-I was provided by isolating the DraIII - BstEII fragment (approximately 45 bp) from another IGF-I expression plasmid, pLS331amB. The variations in the nucleotide sequence for the STII signal were derived from the two strands of synthetic DNA listed below:

```
5'- GCATGTCTAGAATT ATG AAR AAR AAY ATH GCN TTY CTN CTN GCN TCN ATG TTY

GTN TTY TCN ATH GCT ACA AAC GCG TAT GCC ACTCT -3'                   (SEQ ID NO:1)

            3'- CGA TGT TTG CGC ATA CGG TGAGACACGCCACGACTT - 5'      (SEQ ID NO:2)

R: A, G
Y: T, C
H: A, T, C
N: G, A, T, C
```

These two strands of synthetic DNA were annealed and treated with DNA Polymerase I (Klenow Fragment) to form duplex DNA of approximately 101 bp. This duplex DNA was then digested with XbaI and DraIII to generate the fragment of approximately 82 bp encoding the STII signal sequence with variable codons and the first two amino acids of des(1,3)-IGF-I. These fragments were then ligated together as shown in FIG. **3** to construct the library, pSTIIBK.

D. Selection of pSTIIBK#131

The plasmid library containing a variable codon bank of the STII signal sequence (pSTIIBK) was screened for improved growth of transformants and increased secretion of IGF-1. Basically, plasmids were transformed into host strain 27C7 (see below) and screened for enhanced ability to grow in a low phosphate medium (see Chang et al., supra) plus carbenicillin (50 μg/ml) based on OD_{600} measurements of cell density. Candidate colonies were tested for increased levels of IGF-1 secretion as follows. Colonies were inoculated into 3–5 ml LB plus carbenicillin (50 μg/ml) and grown at 37° C. with shaking for about 5–15 hours. Cultures were diluted 1:100 into 1–3 ml low phosphate medium plus Carbenicillin (50 μg/ml) and induced for 24 hours shaking at 37° C. The induced cultures were centrifuged in microcentrifuge tubes for 5 minutes. Supernatants were diluted into IGF RIA diluent and stored at −20° C. until assayed. The amount of IGF-1 secreted into the medium was measured by a radioimmunoassay.

The level of expression of IGF-1, as measured by the amount of IGF-1 detected in culture supernatants, was compared for pLS33, pSTIIBK#131, and pSTIIC, in FIG. **4**. The variant #131 consistently improved IGF-1 expression over the "original", or wild-type STII signal sequence. pSTIIC showed some slight improvement in expression over the wild-type sequence. pSTIIBK#131 differed from the wild-type STII in 12 codons and in the deletion of one Shine-Dalgarno sequence. pSTIIC was constructed as described below as a control plasmid having only one Shine-Dalgarno sequence and three codon changes near the extreme 3' end of the signal.

E. Construction of pSTIIC

In pSTIIC the STII Shine-Dalgarno sequence was removed from the plasmid pLS33. In addition, by incorporating silent mutations near the 3' end of the STII signal, an MluI site was engineered into pSTIIC. The identical fragments described for the construction of pSTIIBK (the vector from pLS33 and the approximately 45 bp DraIII - BstEII fragment from pLS331amB) were used for the construction of this plasmid. However, the synthetic DNA differed from that described above for the construction of pSTIIBK. For the construction of pSTIIC, the synthetic DNA coding for the STII signal sequence and the first two amino acids of des(1,3)-IGF-I was as follows:

```
5'- CTAGAATT ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT

    3'- TTAA TAC TTT TTC TTA TAG CGT AAA GAA GAA CGT AGA TAC AAG CAA

                        __MluI__

TTT TCT ATT GCT ACA AAC GCG TAT GCC ACTCT - 3'                     (SEQ ID NO:3)

AAA AGA TAA CGA TGT TTG CGC ATA CGG TG - 5'                        (SEQ ID NO:4)
```

These fragments were ligated together as illustrated in FIG. **5** to construct the plasmid pSTIIC.

F. Construction of pSTIILys

The plasmid pSTIILys contained an STII signal sequence that differs from the signal sequence of pSTIIC by only one nucleotide change at the position of the second codon. This signal sequence was constructed from synthetic DNA and placed in a pBR322-based vector for the expression of the polypeptide RANTES (Schall et al., *J Immunol*141(3) :1018–1025 (1988)). The XbaI - MluI vector fragment for this construction was isolated from the plasmid pBK131Ran (a derivative of the plasmid pSTIIBK#131 with the gene encoding RANTES replacing the gene encoding des(1,3)-IGF-I). This vector contained the PhoA promoter, Trp Shine-Dalgarno sequence, the last three amino acids of the STIIC signal sequence and the gene encoding the polypeptide RANTES. As illustrated in FIG. **6**, this fragment was then ligated with the following strands of synthetic DNA to construct the plasmid pSTIILys (SEQ ID NO:3):

```
5'- CTAGAATT ATG AAG AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT
     3'-TTAA TAC TTC TTC TTA TAG CGT AAA GAA GAA CGT AGA TAC AAG CAA
TTT TCT ATT GCT ACA AA - 3'                                          (SEQ ID NO:5)
AAA AGA TAA CGA TGT TTG CGC - 5'                                     (SEQ ID NO:6)
```

G. Construction of Alkaline Phosphatase Plasmids

In order to determine a quantitative TIR value for each of the STII signal sequences described, the alkaline phosphatase gene of *E.coli* was used as a reporter gene. In each of these constructions, the PhoA gene was placed downstream of the PhoA promoter, Trp Shine-Dalgarno sequence and a version of the STII signal sequence. The plasmids pPho21, pPho31, pPho41 and pPho51 contained the signal sequences derived from pSTIIC, pLS33, pSTIIBK#131 and pSTIILys, respectively. In the case of pPho3, the construction also contained he STII Shine-Dalgarno region.

H. Construction of pPho21

The vector fragment for the construction of pPho2l was created by digesting pBR322 with EcoRI and BamHI and isolating the largest fragment. The PhoA promoter, Trp Shine-Dalgarno sequence and STIIC signal sequence (amino acids 1–20) were provided by isolating the approximately 484 bp fragment of pCN131Tsc following digestion with EcoRI and MluI. An identical fragment of approximately 484 bp could have also been generated from pSTIIC, a plasmid which has been described previously. The PhoA gene fragment (approximately 1430 bp) encoding amino acids 24–450 of alkaline phosphatase was generated from the plasmid pbO525 following digestion with Bsp1286 and BamHI (Inouye et al., *J Bacteriol*146(2):668–675 (1981)). This Bsp1286 - BamHI fragment also contains approximately 142 bp of SV40 DNA (Fiers et al., *Nature*273:113–120 (1978)) following the termination codon of alkaline phosphatase. Synthetic DNA was used to link the STII signal sequence with the PhoA gene. The sequence of this DNA encoding the last three amino acids of the STIIC signal sequence and amino acids **1–23**of alkaline phosphatase was as follows:

signal sequence (as described for pSTIIC). The last three amino acids of the STIIC signal sequence and the sequence encoding the PhoA gene were provided by digesting the plasmid pPho21 with MluI and BamHI and isolating the approximately 1505 bp fragment. These fragments were ligated together as shown in FIG. **8** to yield pPho31.

J. Construction of pPho41

The vector fragment for the construction of this plasmid was the identical vector described for pPho21. The PhoA promoter, Trp Shine-Dalgarno sequence and STII signal sequence with pSTIIBK#131 codons (amino acids 1–20) were provided by isolating the approximately 484 bp EcoRI–MluI fragment of pNGF131. An identical fragment could have also been generated from pSTIIBK#131. The last three amino acids of the STIIC signal sequence and the sequence encoding the PhoA gene were provided by digesting the plasmid pPho21 with MluI and BamHI and isolating the approximately 1505 bp fragment. As illustrated in FIG. **9**, these three fragments were then ligated together to construct pPho41.

K. Construction of pPho51

The vector fragment for the construction of pPho51 was generated by digesting the plasmid pLS18 with XbaI–BamHI and isolating the largest fragment. The plasmid pLS18 is a derivative of phGH1 (Chang et al., *Gene*55:189–196 (1987)) and an identical vector would have been generated had phGH1 been used in place of pLS18. This XbaI–BamHI vector contains the PhoA promoter and the Trp Shine-Dalgarno sequence. The STII signal sequence (amino acids 1–20) with pSTIILys codons was provided by isolating the approximately 67 bp fragment generated when pSTIILys was digested with XbaI and MluI. The last three amino acids of the STIIC signal sequence and the sequence

```
5'- CGCGTATGCCCGGACACCAGAAATGCCTGTTCTGGAAAACCGGGCTGCTCAGGGCGATATTACTG
    3'- ATACGGGCCTGTGGTCTTTACGGACAAGACCTTTTGGCCCGACGAGTCCCGCTATAATGAC
CACCCGGCGGTGCT - 3'                                                  (SEQ ID NO:7)
GTGGGCCGCC - 5'                                                      (SEQ ID NO:8)
```

In order to facilitate the construction of this plasmid, the synthetic DNA was preligated to the EcoRI–MluI fragment of pCN131Tsc. This preligation generated a new fragment of about 575 bp. As illustrated in FIG. **7**, the fragment generated from the preligation was then ligated together with the other fragments described to construct pPho2.

I. Construction of pPho31

The vector fragment for the construction of this plasmid was the identical vector described for pPho2. The PhoA promoter, Trp Shine-Dalgarno sequence, STII Shine-Dalgarno sequence and STII signal sequence (amino acids 1–20) were generated from pJAL55. The necessary fragment (approximately 496 bp) from pJAL55 was isolated following digestion with EcoRI and MluI. This EcoRI-MluI fragment only differed from the same region of pLS33 by an engineered MluI site starting at amino acid 20 of the STII

encoding the PhoA gene were provided by digesting the plasmid pPho21 with MluI and BamHI and isolating the approximately 1505 bp fragment. A diagram for the construction of pPho51 is given in FIG. **10**.

L. Construction of pSTIICBK

A second variable codon library of the STII signal sequence, pSTIICBK, was constructed. This second codon library was designed only to focus on the codons closest to the met initiation codon of the STII signal sequence. As illustrated in FIG. **11**, pSTIICBK was a pBR322-based plasmid containing the gene encoding the polypeptide RANTES (Schall et al., *J Immunol*141(3):1018–1025 (1988)) under the control of the PhoA promoter and the Trp Shine-Dalgarno sequence. In this plasmid, secretion of RANTES is directed by an STII signal sequence codon library derived from the following two strands of synthetic DNA:

```
5'- GCATGTCTAGAATT ATG AAR AAR AAY ATH GCN TTT CTT CTT GCA TCT ATG TTC

GTT TTT TCT ATT GCT ACA AAC GCG TAT GCC-3'                    (SEQ ID NO:9)

    3'- AGA TAA CGA TGT TTG CGC ATA CGG TGA - 5'              (SEQ ID NO:10)
R: A, G
Y: T, C
H: A, T, C
N: G, A, T, C
```

These two strands of synthetic DNA were annealed and treated with DNA Polymerase I (Klenow Fragment) to form duplex DNA of approximately 86 bp. This duplex DNA was then digested with XbaI and MluI to generate a fragment of approximately 67 bp encoding the first 20 amino acids of the STII signal sequence with variable codons at positions 2–6.

M. Construction of pSTBKPhoA

To increase the number of STII signal sequences available with differing relative TIR strengths, a convenient method of screening the codon library of pSTIICBK was required. The plasmid pSTBKPhoA was constructed as a solution to this problem. In the plasmid pSTBKPhoA, the STII codon library of pSTIICBK was inserted upstream of the PhoA gene and downstream of the PhoA promoter and the Trp Shine-Dalgarno sequence. PhoA activity thus provided a means by which to discriminate between different versions of the STII signal sequences.

The vector fragment for this construction was created by isolating the largest fragment when p131TGF was digested with XbaI and BamHI. An identical vector could have also been generated from phGH1 (Chang et al., *Gene*55:189–196 (1987)). This vector contained the PhoA promoter and the Trp Shine-Dalgarno sequence. The codon library of the STII signal sequence was provided by isolating the approximately 67 bp fragment generated from pSTIICBK following digestion with XbaI and MluI. The last three amino acids of the STIIC signal sequence and the sequence encoding the PhoA gene were provided by digesting pPho2 with MluI and BamHI and isolating the approximately 1505 bp fragment. As illustrated in FIG. **12**, the fragments were then ligated together to construct pSTBKPhoA.

N. Selection of pSTBKPhoA #81, 86, 107, 116

The plasmids pSTBKPhoA #81, 86, 107, 116 were selected from the codon library of pSTBKPhoA based on their basal level PhoA activity (FIG. **13**). As listed in FIG. **14**, each had a different nucleotide sequence encoding the STII signal sequence.

O. Construction of pST116Pho

This version of the STII signal sequence, ST116, combined the double Shine-Dalgarno sequence described by Chang et al. (*Gene* 55:189–196 (1987)) with the codons of the selected STII sequence pSTBKPhoA #116. This signal sequence was initially constructed in a plasmid designed for the secretion of the pro region of NT3 (pNT3PST116) and then was transferred into a plasmid containing the PhoA gene to obtain a relative TIR measurement (pST116Pho).

P. Construction of pNT3PST116

The vector for this construction was generated by digesting the plasmid pLS18 with XbaI and BamHI and isolating the largest fragment. The plasmid pLS18 was a derivative of phGH1 (Chang et al., *Gene*55:189–196 (1987)) and an identical vector could have been generated from phGH1. This XbaI–BamHI vector contained the PhoA promoter and the Trp Shine-Dalgarno sequence. A fragment (approximately 682 bp) containing the last three amino acids of the STII signal sequence and the coding region for amino acids 19–138 of proNT3 (Jones et al., *Proc Natl Acad Sci* 87:8060–8064 (1990)) was generated from the plasmid pNT3P following digestion with MluI and BamHI. The plasmid pNT3P was a pBR322-based plasmid containing the PhoA promoter, STIIBK#131 version of the STII signal sequence and the coding region for amino acids 19–138 of proNT3. The strands of synthetic DNA listed below provided the sequence for the STII Shine-Dalgarno sequence and the first 20 amino acids of the STII signal sequence:

```
5'- CTAGAGGTTGAGGTGATTTT ATG AAA AAA AAC ATC GCA TTT CTT CTT GCA TCT

    3'- TCCAACTCCACTAAAA TAC TTT TTT TTG TAG CGT AAA GAA GAA CGT AGA
ATG TTC GTT TTT TCT ATT GCT ACA AA - 3'                     (SEQ ID NO:11)

TAC AAG CAA AAA AGA TAA CGA TGT TTG CGC - 5'                (SEQ ID NO:12)
```

These fragments were then ligated together as shown in FIG. **15** to construct pNT3PST116.

Q. Construction of ST116Pho

The vector for the construction of this plasmid was the identical vector described for the construction of pNT3PST116. The STII Shine-Dalgarno sequence and the first 20 amino acids of the STII signal sequence (pSTBKPhoA#116 codons) were generated by isolating the approximately 79 bp fragment from pNT3PST116 following digestion with XbaI and MluI. The last three amino acids of the STIIC signal sequence and the sequence encoding the PhoA gene were isolated from pSTBKPhoA#116 following digestion with MluI and BamHI (approximately 1505 bp fragment). As illustrated in FIG. **16**, ligation of these three fragments resulted in the construction of pST116Pho.

II. Alkaline Phosphatase Assay

In these experiments the altered TIR constructs utilizing the phoA reporter gene were assayed for relative translational strengths by a modification of the method of Amenura et al. (*J. Bacteriol.*152:692–701, 1982).

Basically, the method used was as follows. Plasmids carrying altered sequences, whether in the TIR, the Shine-Dalgarno region, the nucleotide sequence between the Shine Dalgarno region and the start codon of the signal sequence, or the signal sequence itself, whether amino acid sequence variants or nucleotide sequence variants, were used to transform *E. coli* strain 27C7 (ATCC 55,244) although any

PhoA- strain of *E. coli* could be used. Transformant colonies were inoculated into Luria-Bertani medium (LB) plus carbenicillin (50 μg/ml, Sigma, Inc.). Cultures were grown at 37° C. with shaking for 4–8 hr. The equivalent of 1 OD_{600} of each culture was centrifuged, then resuspended in 1 ml strict AP media (0.4% glucose, 20 mM NH_4Cl, 1.6 mM $MgSO_4$, 50 mM KCl, 20 mM NaCl, 120 mM triethanolamine, pH 7.4) plus carbenicillin (50 μg/ml). The mixtures were then immediately placed at −20° C. overnight. After thawing, 1 drop toluene was added to 1 ml of thawed culture. After vortexing, the mixtures were transferred to 16×125 mm test tubes and aerated on a wheel at 37° C. for 1 hr. 40 μl of each toluene treated culture was then added to 1 ml 1 M Tris-HCl pH 8 plus 1 mM PNPP (disodium 4-nitrophenyl phosphate hexahydrate) and left at room temperature for 1 hr. The reactions were stopped by adding 100 ml 1 M sodium phosphate pH 6.5. The OD_{410} was measured within 30 minutes. Enzyme activity was calculated as micromoles of p-nitrophenol liberated per minute per one OD_{600} equivalent of cells.

The results are summarized in Table 1.

TABLE 1

Determination of TIR Relative Strength: Use of PhoA as a Reporter Gene

TIR	PhoA Activity[1]	Standard Deviation	Relative Strength
pBR322	0.0279	0.0069	—
pPho51[2]	0.0858	0.0165	1
pSTBKPhoA#86	0.1125	0.0246	1
pSTBKPhoA#107	0.1510	0.0267	2
pPho41[3]	0.1986	0.0556	3
pSTBKPhoA#81	0.2796	0.0813	4
pPho21[4]	0.4174	0.1145	7
pSTBKPhoA#116	0.5314	0.1478	9
pPho31[5]	0.5396	0.0869	9
pST116Pho	0.7760	0.1272	13

[1]micromoles of p-nitrophenol/min/$O.D._{600}$ cells
[2]same STII variant as pSTIILys
[3]same STII variant as pSTIIBK#131
[4]same STII variant as pSTIIC
[5]wild-type STII + MluI site, last codon GCC.

III. Secretion of Heterologous Polypeptide Examples

The plasmids used in these examples were all very similar in design as described above. Rather than describe in detail each construction, the expression plasmids are described here in general terms. Although a different polypeptide of interest was expressed in each example, the only significant variation between these constructions was the nucleotide sequence following the 3' end of each coding region. Thus, for descriptive purposes, these plasmids were loosely grouped into the following two categories based on their 3' sequence:

Category A: Within about 25 bp 3' to the termination codon of each gene of interest began the sequence encoding the transcriptional terminator described by Scholtissek and Grosse (*Nucleic Acids Res.*15(7):3185 (1987)) followed by the tetracycline resistance gene of pBR322 (Sutcliffe, *Cold Spring Harb Symp Quant Biol* 43:77–90 (1978)). Examples in this category included plasmids designed for the secretion of mature NGF (Ullrich et al., *Nature* 303:821–825 (1983)), mature TGF-β1 (Derynck et al., *Nature* 316:701–705 (1985)) and domains 1 and 2 of ICAM-1 (Staunton et al., *Cell* 52:925–933 (1988)). A schematic representation of these plasmids is given in FIG. **17**.

Category B: Examples in this category included plasmids designed for the secretion of mature VEGF (Leung et al., *Science* 246:1306–1309 (1989)), mature NT3 (Jones et al., *Proc. Natl. Acad. Sci. U.S.A.*87:8060–8064 (1990), RANTES (Schall et al., *J Immunol* 141(3):1018–1025 (1988)), and PhoA. The termination codon in each of these plasmids is followed in the 3' direction by a segment of untranslated DNA (VEGF: approximately 43 bp; mature NT3: approximately 134 bp; RANTES: approximately 7 bp; PhoA: approximately 142 bp). Following this 3' untranslated region, the sequence of pBR322 was re-initiated beginning with either the HindIII site (as in the mature NT3 secretion plasmid) or the BamHI site (PhoA, VEGF, RANTES secretion plasmids). A schematic representation of the plasmids included in this category is illustrated in FIG. **18**.

These plasmids were used to transform the host *E.coli* strain 27C7. Transformant colonies were inoculated into 3–5 ml LB +carbenicillin (50 μg/ml). The cultures were grown at 37° C. with shaking for 3–8 hours. The cultures were then diluted 1:100 into 3 ml low phosphate medium (Chang et al., supra) and grown for about 20 hours with shaking at 37° C. For each culture, an 0.5 OD_{600} aliquot was centrifuged in a microfuge tube.

Each 0.5 OD_{600} pellet was then prepared for gel analysis as follows. Each pellet was resuspended in 50 μl TE (10 mM Tris pH7.6, 1 mM EDTA). After the addition of 10 μl 10% SDS, 5 μl reducing agent (1M dithiothreitol or 1M β-mercaptoethanol), the samples were heated at about 90° C. for 2 minutes and then vortexed. Samples were allowed to cool to room temperature, after which 500 μl acetone was added.

The samples were vortexed and then left at room temperature for about 15 minutes. Samples were centrifuged for 5 minutes. The supernatants were discarded, and the pellets resuspended in 20 μl water, 5 μl reducing agent, 25 μl NOVEX 2X sample buffer. Samples were heated at about 90° C. for 3–5 minutes, then vortexed. After centrifugation for 5 minutes, supernatants were transferred to clean tubes and the pellets discarded. 5–10 μl of each sample was loaded onto 10 well, 1.0 mm NOVEX manufactured gel (San Diego, Calif.) and electrophoresed for 1.5–2 hr at 120 volts. Gels were stained with Coomassie blue to visualize polypeptide (FIGS. **19**–**21**).

To provide further quantitation of the results, some gels were analyzed by densitometry. These are displayed in Table 2 below. Both the polypeptide gels and the densitometry results indicated that the heterologous polypeptides tested were consistently secreted more efficiently when an STII variant of reduced translational strength was used to direct secretion of that polypeptide.

TABLE 2

Examples of Improved Polypeptide Secretion By TIR Modification: Densitometer Scans of Polypeptide Gels

Polypeptide	TIR (Relative Strength)	Amount Secreted (% total host polypeptide)
VEGF	9	0.6
	3	5.9
NGF	9	1.6
	7	1.8
	4	5.7
	1	5.5
RANTES	9	0.3
	9	0.2
	7	0.4

TABLE 2-continued

Examples of Improved Polypeptide Secretion By TIR Modification: Densitometer Scans of Polypeptide Gels

Polypeptide	TIR (Relative Strength)	Amount Secreted (% total host polypeptide)
	4	3.9
	3	3.6
	2	3.5
	1*	1.6
	1	1.7
TGF-β1	7	1.7
	3	9.2

*pSTBKPhoA#86 signal sequence

SEQUENCE LISTING

```
(1) GENERAL INFORMATION:

   (iii) NUMBER OF SEQUENCES: 23


(2) INFORMATION FOR SEQ ID NO:1:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 88 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCATGTCTAG AATTATGAAR AARAAYATHG CNTTYCTNCT NGCNTCNATG                50

TTYGTNTTYT CNATHGCTAC AAACGCGTAT GCCACTCT                              88


(2) INFORMATION FOR SEQ ID NO:2:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 36 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTCAGCACCG CACAGAGTGG CATACGCGTT TGTAGC                                36


(2) INFORMATION FOR SEQ ID NO:3:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 82 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTAGAATTAT GAAAAAGAAT ATCGCATTTC TTCTTGCATC TATGTTCGTT                50

TTTTCTATTG CTACAAACGC GTATGCCACT CT                                    82


(2) INFORMATION FOR SEQ ID NO:4:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 75 bases
          (B) TYPE: nucleic acid
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-continued

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          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTGGCATACG CGTTTGTAGC AATAGAAAAA ACGAACATAG ATGCAAGAAG          50

AAATGCGATA TTCTTTTTCA TAATT                                     75


(2) INFORMATION FOR SEQ ID NO:5:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 67 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTAGAATTAT GAAGAAGAAT ATCGCATTTC TTCTTGCATC TATGTTCGTT          50

TTTTCTATTG CTACAAA                                              67


(2) INFORMATION FOR SEQ ID NO:6:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 67 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCGTTTGTA GCAATAGAAA AAACGAACAT AGATGCAAGA AGAAATGCGA          50

TATTCTTCTT CATAATT                                              67


(2) INFORMATION FOR SEQ ID NO:7:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 79 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGCGTATGCC CGGACACCAG AAATGCCTGT TCTGGAAAAC CGGGCTGCTC          50

AGGGCGATAT TACTGCACCC GGCGGTGCT                                 79


(2) INFORMATION FOR SEQ ID NO:8:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 71 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCGCCGGGTG CAGTAATATC GCCCTGAGCA GCCCGGTTTT CCAGAACAGG          50

CATTTCTGGT GTCCGGGCAT A                                         71


(2) INFORMATION FOR SEQ ID NO:9:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 83 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
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GCATGTCTAG AATTATGAAR AARAAYATHG CNTTTCTTCT TGCATCTATG          50

TTCGTTTTTT CTATTGCTAC AAACGCGTAT GCC                            83


(2) INFORMATION FOR SEQ ID NO:10:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 27 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AGTGGCATAC GCGTTTGTAG CAATAGA                                   27


(2) INFORMATION FOR SEQ ID NO:11:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 79 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CTAGAGGTTG AGGTGATTTT ATGAAAAAAA ACATCGCATT TCTTCTTGCA          50

TCTATGTTCG TTTTTTCTAT TGCTACAAA                                 79


(2) INFORMATION FOR SEQ ID NO:12:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 79 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGCGTTTGTA GCAATAGAAA AAACGAACAT AGATGCAAGA AGAAATGCGA          50

TGTTTTTTTT CATAAAATCA CCTCAACCT                                 79


(2) INFORMATION FOR SEQ ID NO:13:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 506 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: double
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC          50

TCATTGCTGA GTTGTTATTT AAGCTTGCCC AAAAAGAAGA AGAGTCGAAT         100

GAACTGTGTG CGCAGGTAGA AGCTTTGGAG ATTATCGTCA CTGCAATGCT         150

TCGCAATATG GCGCAAAATG ACCAACAGCG GTTGATTGAT CAGGTAGAGG         200

GGGCGCTGTA CGAGGTAAAG CCCGATGCCA GCATTCCTGA CGACGATACG         250

GAGCTGCTGC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTCAGTA         300

AAAAGTTAAT CTTTTCAACA GCTGTCATAA AGTTGTCACG GCCGAGACTT         350

ATAGTCGCTT TGTTTTTATT TTTTAATGTA TTTGTAACTA GTACGCAAGT         400

TCACGTAAAA AGGGTATCTA GAGGTTGAGG TGATTTTATG AAAAAGAATA         450

TCGCATTTCT TCTTGCATCT ATGTTCGTTT TTTCTATTGC TACAAATGCC         500
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-continued

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TATGCA                                                                  506


(2) INFORMATION FOR SEQ ID NO:14:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 23 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe
  1               5                  10                  15

Ser Ile Ala Thr Asn Ala Tyr Ala
                 20          23


(2) INFORMATION FOR SEQ ID NO:15:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 90 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: double
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCTAGAGGTT GAGGTGATTT TATGAAAAAG AATATCGCAT TTCTTCTTGC                   50

ATCTATGTTC GTTTTTTCTA TTGCTACAAA YGCSTATGCM                              90


(2) INFORMATION FOR SEQ ID NO:16:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 78 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: double
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TCTAGAATTA TGAAAAAGAA TATCGCATTT CTTCTTGCAT CTATGTTCGT                   50

TTTTTCTATT GCTACAAACG CGTATGCM                                           78


(2) INFORMATION FOR SEQ ID NO:17:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 78 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: double
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TCTAGAATTA TGAAGAAGAA TATTGCGTTC CTACTTGCCT CTATGTTTGT                   50

CTTTTCTATA GCTACAAACG CGTATGCM                                           78


(2) INFORMATION FOR SEQ ID NO:18:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 78 bases
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: double
          (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCTAGAATTA TGAAGAAGAA TATCGCATTT CTTCTTGCAT CTATGTTCGT                   50

TTTTTCTATT GCTACAAACG CGTATGCM                                           78
```

-continued

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCTAGAATTA TGAAAAAAAA CATCGCATTT CTTCTTGCAT CTATGTTCGT 50

TTTTTCTATT GCTACAAACG CGTATGCM 78

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TCTAGAATTA TGAAAAAAAA CATTGCCTTT CTTCTTGCAT CTATGTTCGT 50

TTTTTCTATT GCTACAAACG CGTATGCM 78

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TCTAGAATTA TGAAGAAAAA CATCGCTTTT CTTCTTGCAT CTATGTTCGT 50

TTTTTCTATT GCTACAAACG CGTATGCM 78

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 78 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TCTAGAATTA TGAAAAAGAA CATAGCGTTT CTTCTTGCAT CTATGTTCGT 50

TTTTTCTATT GCTACAAACG CGTATGCM 78

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 90 bases
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TCTAGAGGTT GAGGTGATTT TATGAAAAAA AACATCGCAT TTCTTCTTGC 50

ATCTATGTTC GTTTTTTCTA TTGCTACAAA CGCGTATGCM 90

What is claimed is:

1. A method of optimizing secretion of a heterologous polypeptide of interest in a cell comprising comparing the levels of expression of the polypeptide under control of a set of nucleic acid variants of a translation initiation region, wherein the set of variants represents a range of translational

strengths, and determining the optimal translational strength for production of mature polypeptide, wherein the optimal translational strength is less than the translational strength of the wild-type translation initiation region.

2. The method of claim **1**, wherein the variants comprise nucleic acid variants of a secretion signal sequence.

3. The method of claim **2**, wherein the variant secretion signal sequences are variants of STII.

4. The method of claim **3**, wherein the STII variants are the following variants:

5'-TCTAGAGGTTGAGGTGATTTT ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCS TAT GCM 3'; (SEQ ID NO: 15, wherein Y at position 81 is C)

5'-TCTAGAGGTTGAGGTGATTTT ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAY GCG TAT GCM 3'; (SEQ ID NO: 15, wherein S at position 84 is G)

5'-TCTAGAGGTTGAGGTGATTTT ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAY GCS TAT GCC 3'; (SEQ ID NO:15, wherein M at position 90 is C)

5' TCTAGAATT ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCG TAT GCM 3' (SEQ ID NO:16);

5' TCTAGAATT ATG AAG AAG AAT ATT GCG TTC CTA CTT

-continued

GCC TCT ATG TTT GTC TTT TCT ATA GCT ACA AAC GCG TAT GCM 3' (SEQ ID NO:17);

5' TCTAGAATT ATG AAG AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCG TAT GCM 3' (SEQ ID NO:18);

5' TCTAGAATT ATG AAA AAA AAC ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCG TAT GCM 3' (SEQ ID NO:19);

5' TCTAGAATT ATG AAA AAA AAC ATT GCC TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCG TAT GCM 3' (SEQ ID NO:20);

5' TCTAGAATT ATG AAG AAA AAC ATC GCT TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCG TAT GCM 3' (SEQ ID NO:21);

5' TCTAGAATT ATG AAA AAG AAC ATA GCG TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCG TAT GCM 3' (SEQ ID NO:22); and

5' TCTAGAGGTTGAGGTGATTTT ATG AAA AAA AAC ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCG TAT GCM 3' (SEQ ID NO:23).

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,242,177 B1
DATED : June 5, 2001
INVENTOR(S) : Laura C. Simmons and Daniel G. Yansura

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

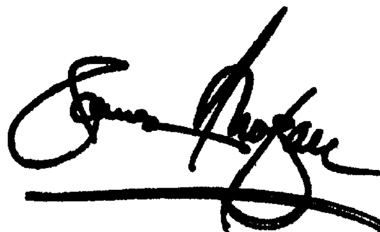
Column 3,
Line 19, please replace "FIG. 14 depicts" with -- FIG. 14A and 14B depict --

Column 11,
Lines 53-54, please replace "FIG. 14" with -- FIG. 14A and 14B --

Signed and Sealed this

Ninth Day of July, 2002

Attest:



JAMES E. ROGAN

Attesting Officer — *Director of the United States Patent and Trademark Office*

EXHIBIT NN

US006121428A

United States Patent [19]

Blank et al.

[11] **Patent Number: 6,121,428**

[45] **Date of Patent: Sep. 19, 2000**

[54] **PROTEIN RECOVERY**

[75] Inventors: **Gregory S. Blank**, Menlo Park; **Daljit S. Narindray**, Pleasanton; **Gerardo A. Zapata**, Foster City, all of Calif.

[73] Assignee: **Genentech, Inc.**, South San Francisco, Calif.

[21] Appl. No.: **09/097,309**

[22] Filed: **Jun. 12, 1998**

Related U.S. Application Data

[60] Provisional application No. 60/050,951, Jun. 13, 1997.

[51] **Int. Cl.**[7] **C07K 1/22**; C07K 1/34

[52] **U.S. Cl.** **530/413**; 435/269; 435/272; 530/344; 530/412; 530/414; 530/417

[58] **Field of Search** 435/68.1, 803, 435/269, 272; 530/387.1, 413, 412, 344, 390.5, 414, 417; 436/518, 824

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,007,113	2/1977	Ostreicher .	
4,305,782	12/1981	Ostreicher et al. .	
4,604,208	8/1986	Chu et al. .	
4,676,980	6/1987	Segal et al. .	
4,816,567	3/1989	Cabilly et al. .	
4,849,352	7/1989	Sullivan et al. .	
5,115,101	5/1992	Bloom et al.	530/413
5,429,746	7/1995	Shadle et al.	530/413
5,470,954	11/1995	Neslund et al.	530/413
5,534,615	7/1996	Baker et al. .	
5,591,828	1/1997	Bosslet et al. .	

FOREIGN PATENT DOCUMENTS

003089	7/1979	European Pat. Off. .
127737	12/1984	European Pat. Off. .
90/11814	10/1990	WIPO .
91/00360	1/1991	WIPO .
92/20373	11/1992	WIPO .
93/08829	5/1993	WIPO .
93/11161	6/1993	WIPO .
WO 93/11162	6/1993	WIPO .
93/16185	8/1993	WIPO .
94/04690	3/1994	WIPO .
96/27011	9/1996	WIPO .
9627011	9/1996	WIPO .

OTHER PUBLICATIONS

Brennan et al., "Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G_1 fragments" *Science* 229:81–83 (Jul. 1985).

Bruggemann et al., "Designer Mice: The Production of Human Antibody Repertoires in Transgenic Animals" *Year in Immunology* 7:33–40 (1993).

Carter et al., "High level escherichia coli expression and production of a bivalent humanized antibody fragment" *Bio/Technology* 10:163–167 (1992).

Carter et al., "Humanization of an anti–p185^{HER2} antibody for human cancer therapy" *Proc. Natl. Acad. Sci.* 89:4285–4289 (1992).

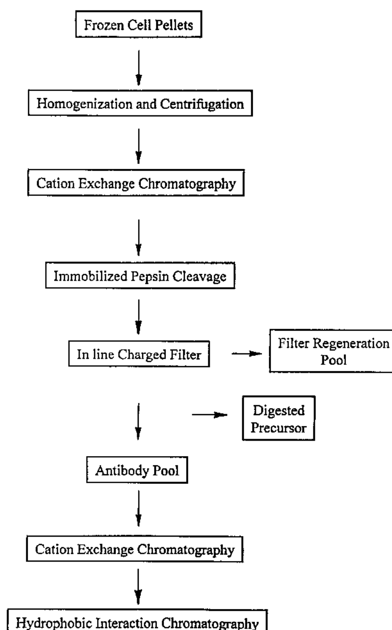
(List continued on next page.)

Primary Examiner—David Saunders
Attorney, Agent, or Firm—Lee K. Tan; Genentech, Inc.

[57] **ABSTRACT**

The invention herein provides a method for recovering a polypeptide comprising exposing a composition comprising a polypeptide to a reagent which binds to, or modifies, the polypeptide, wherein the reagent is immobilized on a solid phase; and then passing the composition through a filter bearing a charge which is opposite to the charge of the reagent in the composition, so as to remove leached reagent from the composition.

16 Claims, 8 Drawing Sheets



OTHER PUBLICATIONS

Clackson et al., "Making antibody fragments using phage display libraries" *Nature* 352:624–628 (1991).

Duchosal et al., "Immunization of hu–PBL–SCID mice and the rescue of human monoclonal Fab fragments through combinatorial libraries" *Nature* 355(6357):258–262 (Jan. 16, 1992).

Gruber et al., "Efficient Tumor Cell Lysis Mediated by a Bispecific Single Chain Antibody Expressed in Escherichia Coli" *Journal of Immunology* 152:5368–5374 (1994).

Holliger et al., ""Diabodies": Small bivalent and bispecific antibody fragments" Proc. Natl. Acad. Sci. USA 90:6444–6448 (Jul. 1993).

Jakobovits et al., "Analysis of Homozygous Mutant Chimeric Mice: Deletion of the Immunoglobulin Heavy–Chain Joining Region Blocks B–cell Development and Antibody Production" *Proc. Natl. Acad. Sci. USA* 90:2551–2555 (Mar. 1993).

Jakobovits et al., "Germ–line Transmission and Expression of a Human–Derived Yeast Artificial Chromosome" *Nature* 362:255–258 (Mar. 18, 1993).

Kostelny et al., "Formation of a Bispecific Antibody by the Use of Leucine Zippers" *Journal of Immunology* 148(5):1547–1553 (1992).

Landschulz et al., "The Leucine Zipper: A Hypothetical Structure Common to a New Class of DNA Binding Proteins" *Science* 240:1759–1764 (1988).

Lindmark et al., "Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera" *J. Immunol. Meth.* 62:1–13 (1983).

Marks et al., "By–passing immunization: building high affinity human antibodies by chain shuffling" *Bio/Technology* 10:779–783 (1992).

Marks et al., "By–passing immunization: human antibodies from V–gene libraries displayed on phage" *J. Mol. Biol.* 222:581–597 (1991).

McCafferty et al., "Phage antibodies: filamentous phage displaying antibody variable domains" *Nature* 348:552–554 (1990).

Milstein et al., "Hybrid Hybridomas and Their Use in Immunohistochemistry" *Nature* 305:537–540 (1983).

Morimoto et al., "Single–step purification of F(ab')2 fragments of mouse monoclonal antibodies (immunoglobulins G1) by hydrophobic interaction high performance liquid chromatography using TSKgel Phenyl–5PW" *Journal of Biochemical and Biophysical Methods* 24:107–117 (1992).

Morrison et al., "Chimeric Human Antibody Molecules: Mouse Antigen–binding Domains with Human Constant Region Domains" *Proc. Natl. Acad. Sci. USA* 81:6851–6855 (Nov. 1984).

O'Shea et al., "Preferential heterodimer formation by isolated leucine zippers from fos and jun" *Science* 245(4918):646–648 (Aug. 11, 1989).

Pluckthun, "Antibodies from *Escherichia coli*" *The Pharmacology of Monoclonal Antibodies*, Rosenburg and Moore, New York:Springer–Verlag, Chapter 11, vol. 113:269–315 (1994).

Presta et al., "Humanization of an Antibody Directed Against IgE" *J. Immunol.* 151(5):2623–2632 (Sep. 1, 1993).

Sahni et al., "Proteosynthetic activity of immobilized Staphylococcus aureus V8 protease: application in the semisynthesis of molecular variants of α–globin" *Analytical Biochemistry* 193(2):178–185 (Mar. 2, 1991).

Shalaby et al., "Development of Humanized Bispecific Antibodies Reactive with Cytotoxic Lymphocytes and Tumor Cells Overexpressing the HER2 Protooncogene" *Journal of Experimental Medicine* 175:217–225 (Jan. 1, 1992).

Suresh et al., "Bispecific Monoclonal Antibodies from Hybrid Hybridomas" *Methods in Enzymology* 121:210–228 (1986).

Traunecker et al., "Bispecific Single Chain Molecules (Janusins) Target Cytotoxic Lymphocytes on HIV Infected Cells" *EMBO Journal* 10(12):3655–3659 (1991).

Tutt et al., "Trispecific F(ab')3 Derivatives that Use Cooperative Signaling Via the TCR/CD3 Complex and CD2 to Activate and Redirect Resting Cytotoxic T Cells" *J. Immunol.* 147(1):60–69 (1991).

Voyksner et al., "Optimization of immobilized enzyme hydrolysis combined with high–performance liquid chromatography/thermospray mass spectrometry for the determination of neuropeptides" *Analytical Biochemistry* 188(1):72–81 (Jul. 1990).

Waterhouse et al., "Combinatorial infection and in vivo recombination: a strategy for making large phage antibody repertoires" *Nucleic Acids Research* 21:2265–2266 (1993).

Zapata et al., "Engineering linear F(ab')2 fragments for efficient production in *Escherichia coli* and enhanced antiproliferative activity" *Protein Engineering* 8(10):1057–1062 (1995).

Morimoto et al. J. Biochem. Biophys. Methods 1992.

Clezardin et al. J. Chromatog. 319 (1985); 67–77.

Reif et al. J. Chromatog. A; 654(1993);29–41.

EVQLVESGGGLVQPGGSLRLSCATSGYTFTEYTMHWMRQAPGKGLEWVAGINPKNG
GTSHNQRFMDRFTISVDKSTSTAYMQMNSLRAEDTAVYYCARWRGLNYGFDVRYFD
VWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK
THTCPPCPAPEL*LGGRMKQLEDKVEELLSKNYHLENEVARLKKLVGER*

FIG. 1A

DIQMTQSPSSLSASVGDRVTITCRASQDINNYLNWYQQKPGKAPKLLIYYTSTLHSGVP
SRFSGSGSGTDYTLTISSLQPEDFATYYCQQGNTLPPTFGQGTKVEIKRTVAAPSVFIFPP
SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST
LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

FIG. 1B

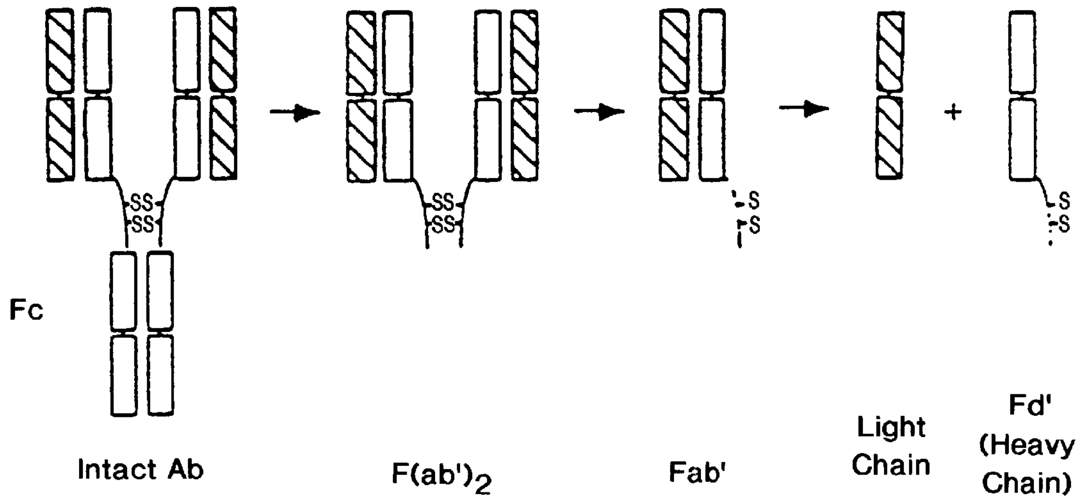


FIG. 2A

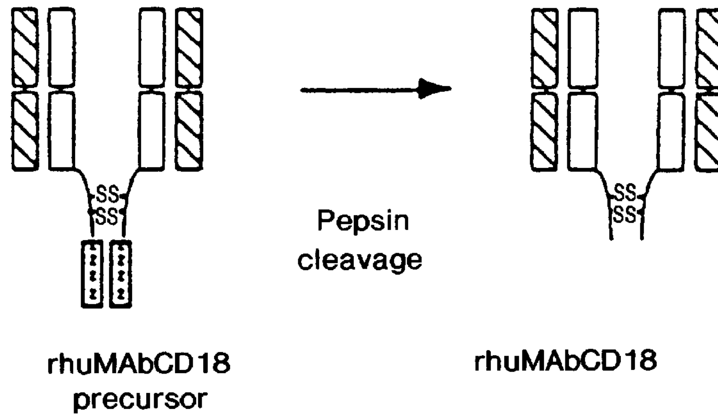


FIG. 2B

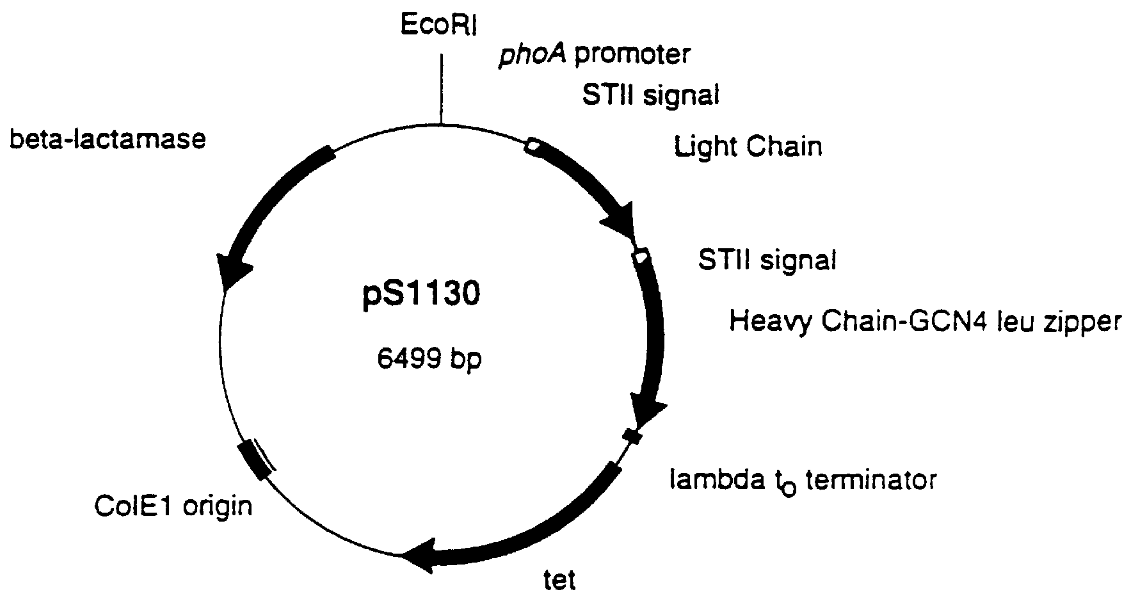


FIG. 3

```
   1 GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC TCATTGCTGA

  61 GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC TTCGCAATAT GGCGCAAAAT

 121 GACCAACAGC GGTTGATTGA TCAGGTAGAG GGGGCGCTGT ACGAGGTAAA GCCCGATGCC

 181 AGCATTCCTG ACGACGATAC GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT

 241 CCTCGTCAGT AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT

 301 TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG CTCGCCGGGG

 361 ATCCTCTAGA GGTTGAGGTG ATTTT ATG AAA AAG AAT ATC GCA TTT CTT CTT
 -23                            M   K   K   N   I   A   F   L   L

 413 GCA TCT ATG TTC GTT TTT TCT ATT GCT ACA AAC GCG TAC GCT GAT ATC
 -14 A   S   M   F   V   F   S   I   A   T   N   A   Y   A   D   I

 461 CAG ATG ACC CAG TCC CCG AGC TCC CTG TCC GCC TCT GTG GGC GAT AGG
   3 Q   M   T   Q   S   P   S   S   L   S   A   S   V   G   D   R

 509 GTC ACC ATC ACC TGT CGT GCC AGT CAG GAC ATC AAC AAT TAT CTG AAC
  19 V   T   I   T   C   R   A   S   Q   D   I   N   N   Y   L   N

 557 TGG TAT CAA CAG AAA CCA GGA AAA GCT CCG AAA CTA CTG ATT TAC TAT
  35 W   Y   Q   Q   K   P   G   K   A   P   K   L   L   I   Y   Y

 605 ACC TCC ACC CTC CAC TCT GGA GTC CCT TCT CGC TTC TCT GGT TCT GGT
  51 T   S   T   L   H   S   G   V   P   S   R   F   S   G   S   G

 653 TCT GGG ACG GAT TAC ACT CTG ACC ATC AGC AGT CTG CAA CCG GAG GAC
  67 S   G   T   D   Y   T   L   T   I   S   S   L   Q   P   E   D

 701 TTC GCA ACT TAT TAC TGT CAG CAA GGT AAT ACT CTG CCG CCG ACG TTC
  83 F   A   T   Y   Y   C   Q   Q   G   N   T   L   P   P   T   F

 749 GGA CAG GGC ACG AAG GTG GAG ATC AAA CGA ACT GTG GCT GCA CCA TCT
  99 G   Q   G   T   K   V   E   I   K   R   T   V   A   A   P   S

 797 GTC TTC ATC TTC CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA ACT GCC
 115 V   F   I   F   P   P   S   D   E   Q   L   K   S   G   T   A

 845 TCT GTT GTG TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG GCC AAA GTA
 131 S   V   V   C   L   L   N   N   F   Y   P   R   E   A   K   V

 893 CAG TGG AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC TCC CAG GAG AGT
 147 Q   W   K   V   D   N   A   L   Q   S   G   N   S   Q   E   S

 941 GTC ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC
 163 V   T   E   Q   D   S   K   D   S   T   Y   S   L   S   S   T

 989 CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC TGC
 179 L   T   L   S   K   A   D   Y   E   K   H   K   V   Y   A   C

1037 GAA GTC ACC CAT CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC TTC AAC
 195 E   V   T   H   Q   G   L   S   S   P   V   T   K   S   F   N

1085 AGG GGA GAG TGT TAA  G CTGATCCTCT ACGCCGGACG CATCGTGGCG
 211 R   G   E   C
```

FIG. 4A

```
1131 CTAGTACGCA AGTTCACGTA AAAACGGTAT CTAGAGGTTG AGGTGATTTT  ATG AAA
 -23                                                        M   K

1187 AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT
 -21 K   N   I   A   F   L   L   A   S   M   F   V   F   S   I   A

1235 ACA AAC GCG TAC GCT GAG GTT CAG CTG GTG GAG TCT GGC GGT GGC CTG
  -5 T   N   A   Y   A   E   V   Q   L   V   E   S   G   G   G   L

1283 GTG CAG CCA GGG GGC TCA CTC CGT TTG TCC TGT GCA ACT TCT GGC TAC
  12 V   Q   P   G   G   S   L   R   L   S   C   A   T   S   G   Y

1331 ACC TTT ACC GAA TAC ACT ATG CAC TGG ATG CGT CAG GCC CCG GGT AAG
  28 T   F   T   E   Y   T   M   H   W   M   R   Q   A   P   G   K

1379 GGC CTG GAA TGG GTT GCA GGG ATT AAT CCT AAA AAC GGT GGT ACC AGC
  44 G   L   E   W   V   A   G   I   N   P   K   N   G   G   T   S

1427 CAC AAC CAG AGG TTC ATG GAC CGT TTC ACT ATA AGC GTA GAT AAA TCC
  60 H   N   Q   R   F   M   D   R   F   T   I   S   V   D   K   S

1475 ACC AGT ACA GCC TAC ATG CAA ATG AAC AGC CTG CGT GCT GAG GAC ACT
  76 T   S   T   A   Y   M   Q   M   N   S   L   R   A   E   D   T

1523 GCC GTC TAT TAT TGT GCT AGA TGG CGA GGC CTG AAC TAC GGC TTT GAC
  92 A   V   Y   Y   C   A   R   W   R   G   L   N   Y   G   F   D

1571 GTC CGT TAT TTT GAC GTC TGG GGT CAA GGA ACC CTG GTC ACC GTC TCC
 108 V   R   Y   F   D   V   W   G   Q   G   T   L   V   T   V   S

1619 TCG GCC TCC ACC AAG GGC CCA TCG GTC TTC CCC CTG GCA CCC TCC TCC
 124 S   A   S   T   K   G   P   S   V   F   P   L   A   P   S   S

1667 AAG AGC ACC TCT GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC AAG GAC
 140 K   S   T   S   G   G   T   A   A   L   G   C   L   V   K   D

1715 TAC TTC CCC GAA CCG GTG ACG GTG TCG TGG AAC TCA GGC GCC CTG ACC
 156 Y   F   P   E   P   V   T   V   S   W   N   S   G   A   L   T

1763 AGC GGC GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC TCA GGA CTC TAC
 172 S   G   V   H   T   F   P   A   V   L   Q   S   S   G   L   Y

1811 TCC CTC AGC AGC GTG GTG ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG
 188 S   L   S   S   V   V   T   V   P   S   S   S   L   G   T   Q

1859 ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC AGC AAC ACC AAG GTC GAC
 204 T   Y   I   C   N   V   N   H   K   P   S   N   T   K   V   D

1907 AAG AAA GTT GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCG CCG
 220 K   K   V   E   P   K   S   C   D   K   T   H   T   C   P   P

1955 TGC CCA GCA CCA GAA CTG CTG GGC GGC CGC ATG AAA CAG CTA GAG GAC
 236 C   P   A   P   E   L   L   G   G   R   M   K   Q   L   E   D

2003 AAG GTC GAA GAG CTA CTC TCC AAG AAC TAC CAC CTA GAG AAT GAA GTG
 252 K   V   E   E   L   L   S   K   N   Y   H   L   E   N   E   V

2051 GCA AGA CTC AAA AAG CTT GTC GGG GAG CGC TAA  GCATGCG ACGGCCCTAG
 268 A   R   L   K   K   L   V   G   E   R

2101 AGTCCCTAAC GCTCGGTTGC CGCCGGGCGT TTTTTATTGT TAA
```

FIG. 4B

Strain	Genotype
W3110	K-12 F⁻ lambda⁻ IN*rrnD-rrnE1*
↓	
1A2	W3110 Δ*fhuA*
↓	
7C1	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*)
↓	
16C9	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*) *deoC*
↓	
23E3	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*) *deoC* Δ*degP*
↓	
33B6	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*) *deoC* Δ*degP* *ilvG*
↓	
49B2	W3110 Δ*fhuA* Δ*phoA* Δ(*argF-lac*) *deoC* Δ*degP* *ilvG* Δ*fucP*
↓	
49A5	W3110 ΔfhuA ΔphoA Δ(*argF-lac*) *deoC* ΔdegP *ilvG* ΔfucP ΔmalE

FIG. 5

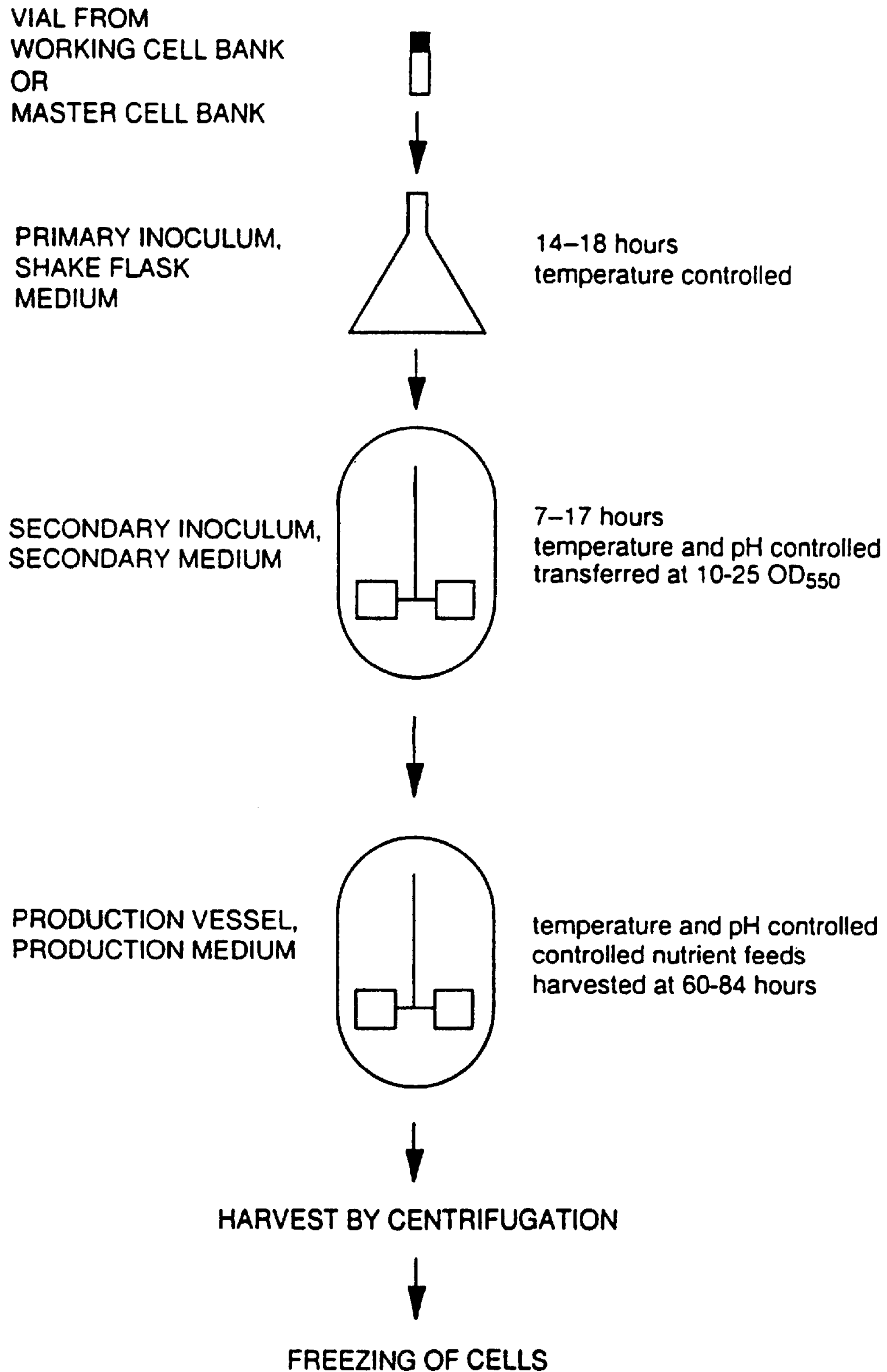


FIG. 6

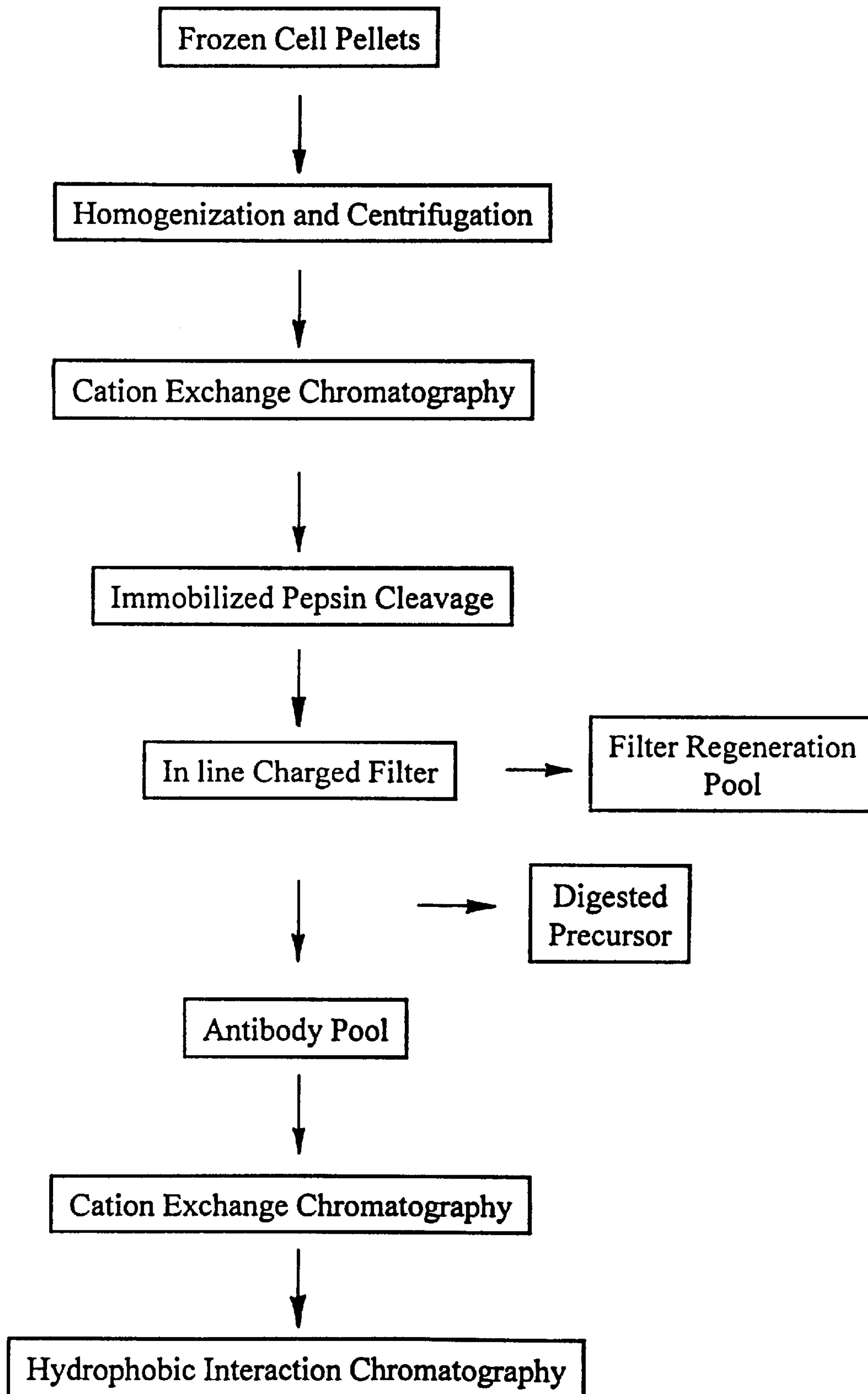


FIG. 7

PROTEIN RECOVERY

RELATED APPLICATION

This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional application number 60/050,951 filed Jun. 13, 1997, the contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates generally to protein recovery. In particular, it pertains to recovery of a polypeptide, wherein the polypeptide is exposed to an immobilized reagent which binds to, or modifies, the polypeptide.

2. Description of Related Art

The large-scale, economic purification of proteins is increasingly an important problem for the biotechnology industry. Generally, proteins are produced by cell culture, using either mammalian or bacterial cell lines engineered to produce the protein of interest by insertion of a recombinant plasmid containing the gene for that protein. Since the cell lines used are living organisms, they must be fed with a complex growth medium, containing sugars, amino acids, and growth factors, usually supplied from preparations of animal serum. Separation of the desired protein from the mixture of compounds fed to the cells and from the by-products of the cells themselves to a purity sufficient for use as a human therapeutic poses a formidable challenge.

Procedures for purification of proteins from cell debris initially depend on the site of expression of the protein. Some proteins can be caused to be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For the latter proteins, the first step of a purification process involves lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. The same problem arises, although on a smaller scale, with directly secreted proteins due to the natural death of cells and release of intracellular host cell proteins in the course of the protein production run.

Once a clarified solution containing the protein of interest has been obtained, its separation from the other proteins produced by the cell is usually attempted using a combination of different chromatography techniques. These techniques separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size. Several different chromatography resins are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. The essence of each of these separation methods is that proteins can be caused either to move at different rates down a long column, achieving a physical separation that increases as they pass further down the column, or to adhere selectively to the separation medium, being then differentially eluted by different solvents. In some cases, the desired protein is separated from impurities when the impurities specifically adhere to the column, and the protein of interest does not, that is, the protein of interest is present in the "flow-through."

As part of the overall recovery process for the protein, the protein may be exposed to an immobilized reagent which binds to or modifies the protein. For example, the protein may be subjected to affinity chromatography wherein an immobilized reagent which binds specifically to the protein, such as an antibody, captures the antibody and impurities pass through the affinity chromatography column. The protein can be subsequently eluted from the column by changing the conditions such that the protein no longer binds to the immobilized reagent. The immobilized reagent may also be an enzyme which modifies the protein. Sahni et al., *Anal. Biochem.* 193:178–185 (1991) and Voyksner et al., *Anal. Biochem.* 188:72–81 (1990) describe immobilized proteases.

Another type of purification process is filtration. Filtration of fine particle size contaminants from fluids has been accomplished by the use of various porous filter media through which a contaminated composition is passed such that the filter retains the contaminant. Retention of the contaminant may occur by mechanical straining or electrokinetic particle capture and adsorption. In mechanical straining, a particle is retained by physical entrapment when it attempts to pass through a pore smaller than itself. In the case of electrokinetic capture mechanisms, the particle collides with a surface within the porous filter and is retained on the surface by short range attractive forces. To achieve electrokinetic capture, charge modifying systems can be used to alter the surface charge characteristics of a filter (see, e.g., W090/11814). For example, where the contaminant to be removed is anionic, a cationic charge modifier can be used to alter the charge characteristics of the filter such that the contaminant is retained by the filter.

There is a need in the art for improved methods for recovering polypeptides, especially those polypeptides produced by recombinant techniques.

SUMMARY OF THE INVENTION

Accordingly, the invention provides a method for recovering a polypeptide comprising: (a) exposing a composition comprising a polypeptide to a reagent which binds to, or modifies, the polypeptide, wherein the reagent is immobilized on a solid phase; and then (b) passing the composition through a filter bearing a charge which is opposite to the charge of the reagent in the composition, so as to remove leached reagent from the composition. Preferably the charge characteristics of the polypeptide in the composition in step (b) are such that the polypeptide passes through the filter and preferably the filter is placed in line with the composition exposed to the reagent as in step (a). In one embodiment of the invention, the polypeptide to be treated in step (a) is a precursor polypeptide and the immobilized reagent is a protease (e.g. pepsin) which removes a precursor domain (e.g. a leucine zipper dimerization domain) from the polypeptide.

The invention also provides a method for recovering a polypeptide comprising removing a leached reagent from a composition comprising the polypeptide and the leached reagent by passing the composition through a filter bearing a charge opposite to that of the leached reagent, wherein the leached reagent was previously immobilized on a solid phase.

In yet a further embodiment, the invention provides a method for modifying a precursor antibody comprising a leucine zipper dimerization domain, comprising exposing the precursor antibody to a protease immobilized on a solid phase such that the protease removes the leucine zipper from the precursor antibody. This method optionally further comprises passing the antibody free of the leucine zipper through

a positively charged filter placed in line with antibody which has been exposed to the immobilized protease.

The anti-CD18 purification process is an example of a process in which an immobilized reagent is required to remove a leucine zipper dimerization domain from the anti-CD18 antibody precursor. The antibody precursor is initially purified using ABX cation exchange chromatography before the leucine zipper domain is removed by digestion with pepsin. The amount of pepsin necessary to completely remove the leucine zipper from the antibody precursor is considerable. A ratio of 1 mg of pepsin per 20 mg of antibody is necessary to carry out the digestion over a reasonable period of time. Treatment like this will leave a large amount of pepsin to be removed in the remaining steps of the anti-CD18 purification process (FIG. **7**). Quick removal of pepsin was found to be beneficial, since excessive exposure to pepsin resulted in overdigestion of the anti-CD18 antibody, with significant loses of intact product. In order to effectively control the amount of pepsin added to the anti-CD18 precursor antibody, and effectively eliminate any traces of pepsin that can persist through the purification process, two methods were implemented into the anti-CD18 antibody purification process. First, to considerably reduce the amount of pepsin added to the ABX purified antibody precursor pool, pepsin was immobilized on a solid phase (i.e. coupled to control pore glass beads (CPG) and packed into a column). The digestion reaction was then carried out by flowing the antibody precursor pool through the pepsin-CPG column. This procedure limited the amount of pepsin added into the antibody precursor pool. Nevertheless, a further problem was identified in that pepsin was found to leach from the solid phase. A small amount of pepsin leaching from the solid phase was found to be sufficient to cause overdigestion of the anti-CD18 antibody, resulting in a reduction in product yields. To overcome this problem of pepsin leaching from the solid phase, a positively charged filter was placed in line with the effluent from the pepsin-CPG column. The filter was found to remove all pepsin leaching from the solid phase, thereby preventing overdigestion of the antibody precursor. Pepsin is an acidic protein with a low pl. Therefore at pH 4, the pH of the digestion step, pepsin remained negatively charged and bound strongly to the positively charged filter. The use of a charged filter instead of a resin to remove leachables was found to be advantageous, since filters are compact and capable of very high flow rates with minimal backpressure. A filter can be implemented in line without the need to perform a separate recovery step, therefore reducing process complexity and time.

It is envisaged that negatively and positively charged filters can be used to solve problems associated with leaching of formerly immobilized reagents in other recovery processes.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. **1**A and **1**B depict the amino acid sequence of rhuMAb CD18 heavy chain (FIG. **1**A; SEQ ID NO:1) and light chain (FIG. **1**B; SEQ ID NO:2). The sequence in italics in FIG. **1**A (SEQ ID NO:3) is that of the leucine zipper.

FIGS. **2**A and **2**B depict intact antibody (Ab) and a variety of antibody fragments ($F(ab')_2$, Fab', light chain and Fd'). Heavy chains are depicted in white and light chains are hatched. The two disulfide bonds that form between two heavy chains are shown as -ss-. FIG. **2**B shows pepsin cleavage of the rhuMAb CD18 precursor to yield rhuMAb CD18, free of the leucine zipper.

FIG. **3** depicts the structure of plasmid pS1130 used to produce rhuMAb CD18 of the example below.

FIGS. **4**A and **4**B depict the full sequence of the pS1130 expression cassette (SEQ ID NO:5).

FIG. **5** shows derivation of the 49A5 production cell line.

FIG. **6** is a schematic of the fermentation process for rhuMAb CD18.

FIG. **7** is a flow diagram depicting the purification steps for rhuMAb CD18.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

As used herein, "polypeptide" refers generally to peptides and proteins having more than about ten amino acids. Preferably, the polypeptide is a mammalian protein, examples of which include renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1–3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins (IGFBPs); CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments and/or variants of any of the above-listed polypeptides.

A "variant" or "amino acid sequence variant" of a starting polypeptide is a polypeptide that comprises an amino acid sequence different from that of the starting polypeptide. Generally, a variant will possess at least 80% sequence identity, preferably at least 90% sequence identity, more

preferably at least 95% sequence identity, and most preferably at least 98% sequence identity with the native polypeptide. Percentage sequence identity is determined, for example, by the Fitch et al., *Proc. Natl. Acad. Sci. USA* 80:1382–1386 (1983), version of the algorithm described by Needleman et al., *J. Mol. Biol.* 48:443–453 (1970), after aligning the sequences to provide for maximum homology. Amino acid sequence variants of a polypeptide are prepared by introducing appropriate nucleotide changes into DNA encoding the polypeptide, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequence of the polypeptide of interest. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the polypeptide, such as changing the number or position of glycosylation sites. Methods for generating amino acid sequence variants of polypeptides are described in U.S. Pat. No. 5,534,615, expressly incorporated herein by reference, for example.

In preferred embodiments of the invention, the polypeptide is a recombinant polypeptide. A "recombinant polypeptide" is one which has been produced in a host cell which has been transformed or transfected with nucleic acid encoding the polypeptide, or produces the polypeptide as a result of homologous recombination. "Transformation" and "transfection" are used interchangeably to refer to the process of introducing nucleic acid into a cell. Following transformation or transfection, the nucleic acid may integrate into the host cell genome, or may exist as an extrachromosomal element. The "host cell" includes a cell in in vitro cell culture as well a cell within a host animal. Methods for recombinant production of polypeptides are described in U.S. Pat. No. 5,534,615, expressly incorporated herein by reference, for example.

A "precursor polypeptide" herein is a polypeptide to which is fused one or more precursor domains, e.g. where the precursor domain is part of a polypeptide chain of the polypeptide or is covalently attached to the polypeptide by a chemical linker, for example. The "precursor domain" may be an amino acid residue or polypeptide. For example, the precursor domain may be a dimerization domain such as a leucine zipper, an amino acid sequence such as polyglutamic acid which bears a negative charge and another amino acid sequence such as polylysine which bears a positive charge, or a peptide helix bundle comprising a helix, a turn and another helix; an epitope tag useful, e.g., in purification of the polypeptide of interest; an amino acid residue or peptide at the amino or carboxy terminus of the polypeptide which is desired to be removed to generate a homogenous polypeptide preparation; a N-terminal methionine, an artifact of production of the polypeptide in recombinant cell culture; a pre, pro or prepro domain of a mature polypeptide (e.g. the pro domain of prothrombin, wherein removal of the pro domain generates the biologically active mature thrombin molecule); a polylysine polypeptide; an enzyme such as glutathione transferase; or the Fc region of an intact antibody which is removed to generate an $F(ab')_2$.

An "epitope tag" polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with activity of the polypeptide to which it is fused. The epitope tag preferably is sufficiently unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable epitope tag polypeptides generally have at least 6 amino acid residues and usually between about 8–50 amino acid residues (preferably between about 914 30 residues). Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field et al. *Mol. Cell. Biol.* 8:2159–2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan et al., *Mol. Cell. Biol.* 5(12):3610–3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky et al., *Protein Engineering* 3(6):547–553 (1990)).

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

The antibody herein is directed against an "antigen" of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those polypeptides discussed above. Preferred molecular targets for antibodies encompassed by the present invention include CD polypeptides such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150,95, VLA4, ICAM-1, VCAM and $\alpha v/\beta 3$ integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA4; polypeptide C etc. Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). In a further embodiment, "monoclonal antibodies" can be isolated from

antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552–554 (1990). Clackson et al, *Nature,* 352:624–628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581–597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779–783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al, *Nuc. Acids. Res.,* 21:2265–2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies. Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255–258 (1993); Bruggemann et al., *Year in Immuno.,* 7:33 (1993); and Duchosal et al. *Nature* 355:258 (1992).

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851–6855 (1984)).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (i.e. residues 24–34 (L1), 50–56 (L2) and 89–97 (L3) in the light chain variable domain and (H1), 50–65 (H2) and 95–102 (H3) in the heavy chain variable domain; Kabat et al., *Sequences of Polypeptides of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (i.e. residues 26–32 (L1), 50–52 (L2) and 91–96 (L3) in the light chain variable domain and 26–32 (H1), 53–55 (H2) and 96–101 (H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901–917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined. The CDR and FR residues of the H52 antibody of the example below are identified in Eigenbrot et al. *Polypeptides: Structure, Function and Genetics* 18:49–62 (1994).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993); Chothia et al., *J. Mol. Biol.,* 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immnol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

In a preferred embodiment of the invention, the antibody is an antibody fragment which is preferably human or humanized (see above discussion concerning humanized antibodies).

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et

al., *Journal of Biochemical and Biophysical Methods* 24:107–117 (1992) and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163–167 (1992)). In another embodiment as described in the Example below, the $F(ab')_2$ is formed using the leucine zipper GCN4 to promote assembly of the $F(ab')_2$ molecule. According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenburg and Moore eds. Springer-Verlag, N.Y., pp. 269–315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H–V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Holliger et al., *Proc. Natl. Acad. Sci. USA* 90:6444–6448 (1993).

The expression "linear antibodies" when used throughout this application refers to the antibodies described in Zapata et al. *Polypeptide Eng.* 8(10):1057–1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

"Multispecific antibodies" have binding specificities for at least two different epitopes, where the epitopes are usually from different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein. Examples of BsAbs include those with one arm directed against a tumor cell antigen and the other arm directed against a cytotoxic trigger molecule such as anti-FcγRI/anti-CD15, anti-p185^{HER2}/FcγRIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-CD3/anti-p185^{HER2}, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-CAMA1, anti-CD3/anti-CD19, -anti-CD3/MoV18, anti-neural cell adhesion molecule (NCAM)/anti-CD3, anti-folate binding protein (FBP)/anti-CD3, anti-pan carcinoma associated antigen (AMOC-31)/anti-CD3; BsAbs with one arm which binds specifically to a tumor antigen and one arm which binds to a toxin such as anti-saporin/anti-Id-1, anti-CD22/anti-saporin, anti-CD7/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A chain, anti-interferon-α(IFN-α)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid; BsAbs for converting enzyme activated prodrugs such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol); BsAbs which can be used as fibrinolytic agents such as anti-fibrin/anti-tissue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA); BsAbs for targeting immune complexes to cell surface receptors such as anti-low density lipoprotein (LDL)/anti-Fc receptor (e.g. FcγRI, FcγRII or FcγRIII); BsAbs for use in therapy of infectious diseases such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell receptor:CD3 complex/anti-influenza, anti-FcγR/anti-HIV; BsAbs for tumor detection in vitro or in vivo such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-p185^{HER2}/anti-hapten; BsAbs as vaccine adjuvants; and BsAbs as diagnostic tools such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, anti-HRP/anti-FITC, anti-CEA/anti-β-galactosidase. Examples of trispecific antibodies include anti-CD3/anti-CD4/anti-CD3/, anti-CD3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. $F(ab')_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Milstein et al., *Nature,* 305:537–539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93108829, and in Traunecker et al., *EMBO J.,* 10:3655–3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule

provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology,* 121:210 (1986).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO92/20372, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al, *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217–225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547–1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Holliger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444–6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et aL, *J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al. *J. ImmunoL* 147: 60 (1991).

By "recovering a polypeptide" is meant obtaining a polypeptide preparation from a "pre-recovery preparation" by purifying the pre-recovery preparation (see below) or by modifying a precursor polypeptide to generate a form of the polypeptide which is free of the precursor domain.

By "purifying" a composition comprising an polypeptide and one or more contaminants is meant increasing the degree of purity of the polypeptide in the composition by removing (completely or partially) at least one contaminant from the composition. A "purification step" may be part of an overall purification process resulting in an "essentially pure" composition, which is used herein to refer to a composition comprising at least about 90% by weight of the polypeptide of interest, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" herein refers to a composition comprising at least about 99% by weight of polypeptide of interest, based on total weight of the composition.

The "reagent" of interest herein is a compound or composition (preferably a polypeptide) which is able to bind to and/or modify a polypeptide of interest. A "leached" reagent is one which has come free from the solid phase. The reagent may, for example, bind to the polypeptide as is the case for "capture reagents" used in affinity purification methods. Examples of such "capture reagents" include protein A or protein G for capturing polypeptides such as antibodies and immunoadhesins; antibodies which can be used for affinity purification of polypeptides; a ligand binding domain of a receptor for capturing a ligand thereto; a receptor binding domain for capturing a receptor or a fragment thereof binding protein (e.g. IGFBPs such as IGFBP-3 and growth hormone binding proteins (GHBPs)); and immunoadhesins. Alternatively, or in addition, the reagent may modify the polypeptide of interest. For example, the reagent may chemically or physically alter the polypeptide. By "chemical alteration" is meant modification of the polypeptide by, e.g., bond formation or cleavage resulting in a new chemical entity. By "physical alteration" is meant changes in the higher order structure of the polypeptide. Enzymes are examples of reagents which can chemically and/or physically modify the polypeptide. The preferred enzyme is a protease (e.g. for removing one or more precursor domains from a precursor polypeptide). A "proteasel" is an enzyme which can hydrolyze a polypeptide. Examples of proteases include pepsin, cathepsin, trypsin, papain, elastase, carboxypeptidases, aminopeptidases, subtilisin, chymotrypsin, thermolysin, V_8 protease, prolinase and other endo- or exopeptidases.

By "solid phase" is meant a non-aqueous matrix to which a reagent can adhere. The solid phase may be a purification column, a discontinuous phase of discrete particles, a membrane or filter. Examples of materials for forming the solid phase include polysaccharides (such as agarose and

cellulose); and other mechanically stable matrices such as silica (e.g. controlled pore glass), poly(styrenedivinyl) benzene, polyacrylamide, ceramic particles and derivatives of any of the above. In preferred embodiments, the solid phase comprises controlled pore glass beads retained in a column. In certain embodiments, the solid phase is coated with a reagent (such as glycerol) which is intended to prevent nonspecific adherence of contaminants to the solid phase.

The reagent discussed above may be "immobilized" on or in the solid phase by forming a covalent bond between a functional group of the reagent and a reactive group on the surface of the solid phase. In other embodiments, the reagent is "immobilized" on the solid phase by adsorption and ionic binding or may be entrapped in the solid phase, e.g., within cells or lattice type polymers or microcapsules (See Holenberg and Roberts in *Enzymes as Drugs* John Wiley & Sons NY (1981), pages 396–411). The reagent should essentially retain its ability to bind to and/or modify the polypeptide of interest once immobilized to the solid phase. Reagent immobilization may be achieved by matrix activation. Briefly, this generally involves first activating the solid phase by a specific chemical reaction depending on the surface chemistry and then immobilizing the reagent by combining it with the activated solid phase. Activation of the solid phase can involve activation of hydroxyl groups (e.g. cyanogen bromide activation of the solid phase); carboxyl groups (e.g. using N-hydroxybenzotriazole in the presence of a water-soluble carbodiimide); acyl hydrazide (using, e.g., glutaraldehyde to generate aldehyde groups); amines (using, e.g., nitrous acid, phosgene and thiosphosgene, or cyanogen bromide); or acrylonitrile. In another embodiment, the reagent may be immobilized using a cross-linking agent (i.e. the reagent is immobilized indirectly to the solid phase) such as zero-length cross-linkers (e.g. carbodiimide, Woodward's reagent K, chloroformates and carbonyidiimidazole); homobifunctional cross-linkers (e.g. glutaraldehyde, chloroformates and carbonyidiimidazole, heterocyclic halides, divinylsulfone, quinones and transition metal ions); heterobifunctional cross-linkers including, for example, monohalogenacetyl halide, epichlorohydrin as well as amino and thiol group-directed reagents. In yet a further embodiment, the reagent is cross-linked to the solid phase through a carbohydrate chain. To achieve this, the sugar moieties may be first oxidized to aldheydes which form Schiff bases with either ethylenediamine or glycyltyrosine. Sodium borohydride may be used to stabilize the bonds. The derivatized glycoprotein is immobilized to the solid phase. For a review of immobilization techniques, see Wong, S. *Chemistry of Protein Conjugation and Cross-Linking* CRC Press Inc., Boston (1991).

A "leucine zipper" is a peptide (often about 20–40 amino acid residues long) having several repeating amino acids, in which every seventh amino acid is a leucine residue. Such leucine zipper sequences form amphipathic α-helices, with the leucine residues lined up on the hydrophobic side for dimer formation. Leucine zippers may have the general structural formula known as the heptad repeat (Leucine-X_1-X_2-X_3,-X_4-X_5-X_6;SEQ ID NO:4)$_n$, where X may be any of the conventional 20 amino acids, but is most likely to be amino acids with tight α-helix forming potential, for example, alanine, valine, aspartic acid, glutamic acid and lysine, and n may be three or greater, although typically n is 4 or 5. Examples of leucine zippers herein include the Fos-Jun leucine zipper (O'Shea et al. *Science* 245:646 (1989)) which may be used for forming heterodimers (e.g. bispecific antibodies); the GCN4 leucine zipper from yeast (Landschulz et al. *Science* 240:1759–1764 (1988)) which may be used for forming homodimers (e.g. monospecific antibodies, as in the example below); and leucine zippers found in other DNA-binding proteins, such as C/EBP and c-myc, as well as variants of any of these.

The term "filter" when used herein refers to a porous filter media through which an aqueous phase can pass but which retains one or more contaminants. The filter can be formed from a variety of materials such as cellulose fibers, including, e.g. cellulose acetate (SARTOBIND™ membrane adsorbers by Sartorius); silica based particulate; fibrous and particulate filter elements; nylon membranes or any combination of these. The filter of interest herein is a "charged filter" (i.e. positively or negatively charged) which means that it bears an overall net positive charge or an overall net negative charge. This may be achieved, for example, by attaching "charge modifying groups" to the filter. Anionic charge modifiers include water soluble polymers having anionic functional groups such as carboxyl, phosphorous, phosphonic, sulfonic groups (U.S. Pat. No. 4,604,208). Cationic charge modifiers include melamine formaldehyde cationic colloid (U.S. Pat. No. 4,007,113), inorganic cationic colloidal silica (U.S. Pat. No. 4,305,782), polyamidopolyamine epichlorohydrin cationic resin, polyamine epichlorohydrin. The filter is preferably one which allows high flow rates, without sacrificing binding capacity (as opposed to bead based columns, for example). Various configurations of the filter are contemplated, such as multilayer modules and spiral wound arrangements.

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. An "equilibration buffer" is that used to prepare a solid phase for loading the polypeptide of interest. The "loading buffer" is that which is used to load the composition comprising the polypeptide and contaminants onto the solid phase. Often, the equilibration and loading buffers are the same. The "elution buffer" is used to elute the polypeptide from the solid phase.

As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the "binding domain" of a heterologous "adhesin" polypeptide (e.g. a receptor, ligand or enzyme) with the effector functions of an immunoglobulin constant domain. Structurally, the immunoadhesins comprise a fusion of the adhesin amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site (antigen combining site) of an antibody (i.e. is "heterologous") and an immunoglobulin constant domain sequence. The immunoglobulin constant domain sequence in the immunoadhesin is preferably derived from γ1, γ2, or γ4 heavy chains since immunoadhesins comprising these regions can be purified by protein A chromatography (Lindmark et al., *J Immunol. Meth.* 62:1–13 (1983)).

The term "ligand binding domain" as used herein refers to any native cell-surface receptor or any. region or derivative thereof retaining at least a qualitative ligand binding of a corresponding native receptor. In a specific embodiment, the receptor is from a cell-surface polypeptide having an extracellular domain which is homologous to a member of the immunoglobulin supergenefamily. Other receptors, which are not members of the immunoglobulin supergenefamily but are nonetheless specifically covered by this definition, are receptors for cytokines, and in particular receptors with tyrosine kinase activity (receptor tyrosine kinases), members of the hematopoietin and nerve growth factor receptor superfamilies, and cell adhesion molecules, e.g. (E-, L- and P-) selectins.

The term "receptor binding domain" is used to designate any native ligand for a receptor, including cell adhesion molecules, or any region or derivative of such native ligand retaining at least a qualitative receptor binding ability of a corresponding native ligand. This definition, among others, specifically includes binding sequences from ligands for the above-mentioned receptors.

Modes for Carrying Out the Invention

The invention herein provides a method for modifying a polypeptide and/or purifying a polypeptide from a composition comprising the polypeptide and one or more contaminants. The composition is generally one resulting from the recombinant production of the polypeptide, but may be that resulting from production of the polypeptide by peptide synthesis (or other synthetic means) or the polypeptide may be purified from a native source of the polypeptide. Preferably the polypeptide is an antibody, e.g. one which binds the CD18 antigen.

For recombinant production of the polypeptide, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the polypeptide is readily isolated and sequenced using conventional procedures (e.g., where the polypeptide is an antibody by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence (e.g. as described in U.S. Pat. No. 5,534,615, specifically incorporated herein by reference).

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as Escherichia, e.g., *E. coli*, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., *Salmonella typhimurium*, Serratia, e.g., *Serratia marcescans*, and Shigella, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published Apr. 12, 1989), Pseudomonas such as *P. aeruginosa*, and Streptomyces. One preferred *E. coli* cloning host is *E. coil* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coil* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; Kluyveromyces hosts such as, e.g., *K. lactis, K. fragilis* (ATCC 12,424), *K bulgaricus* (ATCC 16,045), *K wickeramii* (ATCC 24,178), *K waltii* (ATCC 56,500), *K drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus*; yarrowia (EP 402,226); *Pichia pastoris* (EP 183,070); Candida; *Trichoderma reesia* (EP 244,234); *Neurospora crassa*; Schwanniomyces such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., Neurospora, Penicillium, Tolypocladium, and Aspergillus hosts such as *A. nidulans* and *A. niger.*

Suitable host cells for the expression of glycosylated polypeptide are derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243–251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y Acad. Sci.* 383:44–68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The host cells used to produce the polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al., *Meth. Enz.* 58:44 (1979), Barnes et al., *Anal. Biochem.*102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. No. Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

When using recombinant techniques, the polypeptide can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the polypeptide is produced intracellularly, as a first step, the particulate debris, either host cells or lysed cells (e.g. resulting from homogenization), is removed, for example, by centrifugation or ultrafiltration. Where the polypeptide is secreted into the medium, supernatants from such expression systems are generally first concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit.

The polypeptide is then subjected to one or more purification steps. Examples of purification procedures include fractionation on an ion-exchange column, hydrophobic interaction chromatography (e.g. on phenyl sepharose), ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin Sepharosem™, anion exchange chromatography, cation exchange chromatography (e.g. on a Bakerbond ABX column or SP-Sepharose HP column), chromatofocusing, SDS-PAGE, ammonium sulfate precipitation, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography (e.g. using protein A, protein G, an antibody, a specific substrate, ligand or antigen as the capture reagent).

In one embodiment of the invention, the recovery step involves exposing a composition comprising the polypeptide (and optionally one or more contaminants) to a solid phase to which is immobilized a reagent which binds to, or modifies, the polypeptide. This step may be at the start or end or anywhere in a sequence of recovery steps for the polypeptide. In one embodiment, the solid phase is packed in a column and the immobilized reagent captures the polypeptide. In another embodiment, the reagent chemically and/or physically modifies the polypeptide and is immobilized on the solid phase which is, e.g., packed in a column, and the composition is passed through the column. For example, the polypeptide may comprise a precursor domain which the immobilized regent removes as part of the recovery process. In the example below, the precursor polypeptide was an antibody with a leucine zipper dimerization domain which was removed by immobilized pepsin in the recovery process. Following this step, the solid phase (e.g. chromatography column) may be regenerated using techniques applicable for regenerating such a solid phase.

It has been discovered herein that leaching of the immobilized reagent from the solid phase can occur and this can result in decreased yields and/or contamination of the polypeptide preparation following this step. In particular, in the example below, it was found that the pepsin could leach from a column to which it was immobilized and result in digestion of the antibody following removal of the leucine zipper, thereby reducing yields of functional antibody.

In order to obviate this problem, the invention provides a step following exposure of the composition to the immobilized reagent as discussed above. This involves passing the composition comprising the polypeptide and leached reagent (and optionally one or more further contaminants) through a filter bearing a charge which is opposite to the charge of the reagent at the pH of the composition, so as to remove leached reagent from the composition. The filter may be positively charged to remove contaminants that are negatively charged at the pH of the composition, such as acidic proteases, protein A, protein G or other reagents that can leach from affinity columns. Alternatively, the filter may be negatively charged to remove contaminants that are positively charged at the pH of the composition, such as basic proteases. Preferably, the charge characteristics of the polypeptide of interest in the composition passed through the filter are such that the polypeptide is not significantly retained by the filter and passes therethrough. The ability of the leached reagent to bind to the filter and the polypeptide to pass through it varies depending on the pH of the composition passing though the filter. To determine which filter to use (i.e. positively or negatively charged filter), one may investigate the pI of the leached reagent and, optionally, the pI of the polypeptide exposed to the immobilized reagent as discussed above. In one embodiment (e.g. as in the example below), the pH of the composition will be such that the leached reagent and polypeptide already have opposite net charges. In another embodiment, it may be beneficial to adjust the pH of the composition to be passed through the charged filter such that the leached reagent and polypeptide have opposite charges. Such alteration of the pH of the composition may serve to increase binding of oppositely charged contaminants to the filter and/or decrease binding of the polypeptide of interest to the filter. Other modifications of the composition to achieve the same effect are envisaged herein. Following any optional modifications of the composition, a filter may be selected which has a charge opposite to that of the leached reagent to be removed from the composition.

In a preferred embodiment of the invention, the filter is placed “in line” with the effluent treated as in the previous step (i.e. the effluent flows directly though the filter). This can be achieved by connecting the filter directly to the column effluent port, before the effluent is collected into a pool tank. The filter may be regenerated using techniques applicable to the type of filter used.

The polypeptide preparation may be subjected to additional purification, if necessary. Exemplary further purification steps have been discussed above. The polypeptide thus recovered may be formulated in a pharmaceutically acceptable carrier and is used for various diagnostic, therapeutic or other uses known for such molecules.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of ail citations in the specification are expressly incorporated herein by reference.

EXAMPLE

This example concerns an antibody (rhuMAb CD18) produced as a precursor polypeptide with a leucine zipper domain which is removed during the purification process of the instant invention. Recombinant humanized anti-CD18 antibody (rhuMAb CD18) having the amino acid sequence shown in FIG. **1**A (heavy chain; SEQ ID NO:1) and FIG. **1**B (light chain; SEQ ID NO:2) was created by humanization of the murine monoclonal antibody muMAb H52 (Hildreth et al. *J. Immunology* 134:3272–3280 (1985)).

Recombinant production of rhuMAb CD18: Plasmid pS1130 was constructed to direct production of the rhuMAb CD18 precursor molecule in *E. coli*. The precursor is cleaved during the purification process by the protease pepsin to yield rhuMAb CD18. rhuMAb CD18 is an F(ab')$_2$ molecule composed of 2 different peptides (light and heavy chains) linked by disulfide bonds. The Fc region of intact antibodies normally holds the 2 Fab arms together (FIG. **2**A), so when Fab' is produced in *E. coli* very little F(ab')$_2$ is formed. Fusion of a yeast GCN4 leucine zipper dimerization domain to the C-terminus of an Fab' substitutes for the Fc region and allows for efficient F(ab')$_2$ production in *E. coli*. The GCN4 leucine zipper domains interact to form

stable dimeric structures (parallel coiled coils) that hold the hinge region cysteine residues of two heavy chains together so that the two native interchain disulfide bonds can form. This results in formation of $F(ab')_2$ complexes that are covalently linked by disulfide bonds. The leucine zipper domains are later removed from the rhuMAb CD18 precursor during the purification process using the protease pepsin, which cleaves uniformly between the 2 leucine residues of the hinge. This results in the formation of the rhuMAb CD18 $F(ab')_2$ molecule (FIG. **2**B).

Plasmid pS1130 (FIG. **3**) is based on the well characterized plasmid pBR322 with a 2143 bp expression cassette (FIG. **4**) inserted into the EcoRI restriction site. Plasmid pS1130 is resistant to both tetracycline and β-lactam antibiotics. The expression cassette contains a single copy of each gene linked in tandem. Transcription of each gene into a single dicistronic mRNA is directed by the *E. coli* phoA promoter (Chang et al. *Gene* 44:121–125 (1986)) and ends at the phage lamda t_0 terminator (Scholtissek and Grosse *Nucleic Acids Research* 15:3185 (1987)). Translation initiation signals for each chain are provided by *E. coli* STII (heat stable enterotoxin) (Picken et al. *Infection and Immunity* 42:269–275 (1983)) Shine-Dalgarno sequences. Translation of each chain begins with a 23 residue STII signal peptide that directs translocation of the peptides across the cytoplasmic membrane into the periplasmic space (SEQ ID NOs: 6 and 7). The STII signal peptide is then removed by the *E. coli* leader peptidase. The light and heavy chains fold into their native conformations after secretion into the periplasm and associate into the rhuMAb CD18 precursor, a covalently linked $F(ab')_2$ (FIG. **2**B). The leucine zipper domain is cleaved from the precursor during the purification process (see below) to yield rhuMAb CD18 (FIG. **2**B). The cell line used in the production of rhuMAb CD18 is 49A5, derived from *E. coli* cell line W3110 (ATCC 27,325) as shown in FIG. **5**. The fermentation procedure takes place as shown in FIG. **6**. Production of rhuMAb CD18 precursor occurs when the medium becomes depleted in phosphate, typically 30–60 hours after inoculation.

Purification of rhuMAb CD18 precursor from the *E. coli* cell paste was as follows.

Homogenization and Centrifugation: Frozen cell pellets containing anti-CD18 precursor antibody, were dissolved in about 3 volumes of extraction buffer (120 mM MES, 5mM EDTA buffer, pH 6) heated to 30–40° C. This resulted in a suspension with a pH between about 5.4 and 6.5. This suspension was passed twice through a Gaulin homogenizer at 5500 to 6500 psi and kept below 20° C. with a heat exchanger. 5% polyethyleineimine (PEI) (w/v), pH 6 was added to the homogenate to a final concentration of 0.2% PEI. The mixture was incubated for about one hour at 2–8° C. About one volume of extraction buffer (120 mM MES, 5 mM EDTA, pH 6) was added before the solids were removed by centrifugation at 15,280 g. The clear supernatant was conditioned to a conductivity of less than 3 mohms by the addition of cold water.

Ion Exchange Chromatography: The conditioned supernatant was loaded onto a cation exchange column (ABX column; Mallinckrodt Baker, Inc., NJ, USA) equilibrated in 50 mM MES, pH 6.0. The column was washed with the equilibration buffer and the anti-CD18 precursor was eluted with a linear gradient from 50 mM MES, pH 6.0 to 50 mM MES, 100 mM sodium citrate, pH 6.0. The column was monitored by absorbance at 280 nm, and the eluate was collected in fractions. The appropriate fractions were pooled based on analytical cation exchange hydrophobic liquid chromatography (HPLC). After use, the cation exchange column was regenerated using 3.0 M guanidine HCl, 20 mM HEPES buffer, pH 7.4, followed by 1% acetic acid, 120 mM phosphoric acid. The column was stored in 1% acetic acid, 120 mM phosphoric acid.

Precursor digestion: Pepsin (Sigma, MO, USA) was chemically coupled to controlled pore glass (CPG) by Bioprocess Ltd., UK. The CPG was activated with $NaIO_4$ followed by reduction of schiff base formation between CPG and pepsin using $NaBH_3CN$.

The cation exchange anti-CD18 precursor antibody pool of the previous step was diluted with 50 mM MES, 36 mM sodium citrate, pH 4.0 to a concentration of approximately 2 g/L. The pool was then adjusted to pH 4 by addition of 2 M citric acid and flowed through a column containing immobilized pepsin (pepsin-CPG) previously equilibrated with 50 mM MES, 36 mM sodium citrate pH 4.0. This procedure removed the zippers from the hinge region while leaving intact $F(ab')_2$. After use, the pepsin column was regenerated with 0.12% aqueous HCl, pH 1.5 and stored in 100 mM sodium acetate, 150 mM sodium chloride, 0.01% Thimerosal, 50% glycerol, pH 4.5.

Anion exchange filtration: The effluent from the pepsin-CPG column was passed directly in line through an anion exchange Sartobind Q membrane (Sartorius, Goettingen, West Germany). The generated anti-CD18 $F(ab')_2$ antibody flows through the membrane while pepsin and other negatively charge impurities bind strongly to the membrane. The membrane was regenerated using 50 mM MES, 36 mM sodium citrate, 1 M sodium chloride, pH 4.0 and was stored in 0.1 N sodium hydroxide.

Analysis of the digestion reaction: Digestion of the anti-CD18 precursor antibody was analyzed by HPLC cation-exchange chromatography on a BAKERBOND™ carboxy-sulfon (CSX) 50×4.6 mm column (J. T. Baker Phillipsburg, N.J.) maintained at 55° C. The polypeptides were eluted using an increasing linear gradient from pH 6.0 to pH 8.0 at a flow rate of 4 ml/min using a detection wavelength of 280 nm. Buffer A contained 16 mM of each HEPES/PIPES/MES, pH 6.0 and Buffer B contained 16 mM of each HEPES/PIPES/MES, pH 8.0. For the separation of digested and undigested anti-CD18 precursor antibody, a linear gradient was run for 10 min from 40% B to 100% B.

Pepsin analysis: The amount of pepsin leached from the pepsin-CPG column was determined by reverse phase HPLC analysis and by pepsin ELISA analysis.

For HPLC analysis, a TosoHass TSK-Phenyl (7.5×75 mm) column was monitored with 90% solvent A (0.1% TFA in water) and 10% solvent B (0.1% TFA in acetonitrile). Upon 75 μg sample injection, a 30 minute gradient from 10% to 25% solvent B was initiated; the flow rate was 1 ml/min, and the temperature was maintained at 55° C. throughout.

For the ELISA, a sandwich ELISA was performed. Polyclonal goat anti-pepsin antibodies were used to coat a 96-well microtiter plate. Pepsin containing samples and standards were incubated in the coated wells. The sandwich was completed with biotinylated-goat-anti-pepsin. Prior to biotinylation, the second antibodies were affinity purified using CPG-pepsin. The immunological complexes were detected in the plates using streptavidin-alkaline phosphatase and p-nitrophenyl phosphate substrate. Absorbance at 405 nm was measured in a microtiter plate reader. Standards cover the range of 33.3 μg/ml down to 0.5 μg/ml in 2-fold dilutions. Dilutions were made for the samples (pure sample or diluted 1:2, 1:4, and 1:8). Samples were also spiked at the level of 10 μg/ml with pepsin and assayed as

samples. The detection limit of the assay was 1 μg/ml. A 4-parameter logistic curve fit to the data produced an acceptable standard curve.

Cation exchange chromatography: The pool was diluted to give a conductivity of approx. 7 mohms by the addition of water. The pool was applied to a cation exchange column (SP Sepharose High Performance; SPHP) equilibrated in 25 mM MES, 60 mM acetic acid, pH 4.0. The SP Sepharose column was washed with 25 mM MES, 75 mM sodium acetate pH 5.6 and eluted in a linear gradient of 75–110 mM sodium acetate in 25 mM MES pH 5.6. The column eluate was monitored at 280 nm and the eluate fractions were pooled based on analytical ion exchange HPLC. The SP Sepharose column was regenerated in 25 mM MES, 4 mM sodium acetate pH 5.6 followed by a wash with 0.5% sodium hydroxide. The column was stored in 0.1% NaOH.

Hydrophobic Interaction Chromatography (HIC): The pooled fraction from the SP sepharose column was diluted with the addition of 3.0M ammonium sulphate, 25 mM MES pH 6.0 at a ratio of 0.26 liters per liter of pool. This was then passed through a HIC column (phenyl sepharose FF—low substitution) previously equilibrated in 0.625 M ammonium sulphate, 25 mM MES pH 6.0. After loading, the column was washed with the same buffer used in the equilibration and the rhuMAb CD18 eluted in 0.375M ammonium sulphate, 25 mM MES pH 6.0. The eluate was monitored at 280 nm and the fractions are collected based on analytical reversed phase HPLC. The HIC column was regenerated in 25 mM MES, pH 6.0, followed by a wash in 0.5% NaOH. The column was stored in 0.1% NaOH.

Results

Two separate large scale purification runs were performed (see FIG. **7**). The purification process started with *E. coli* cell paste containing anti-CD18 precursor antibody, and completed with the anti-CD18 $F(ab')_2$ lacking the leucine zipper dimerization domain. During both purification runs, digestion of the antibody precursor molecule was performed by passing partially purified anti-CD18 precursor antibody through a pepsin-CPG column. Digestion was monitored by SDS PAGE and analytical cation exchange HPLC. The total amount of pepsin leached from the pepsin-CPG column was determined by measuring pepsin in the digested precursor antibody pool after the CPG-pepsin digestion and filtration step and in the anion exchange membrane regeneration pool. Regeneration of the membrane was performed by eluting pepsin and contaminants attached to the membrane using 50 mM MES, 36 mM sodium citrate, 1 M sodium chloride, buffer pH 4.0 (see FIG. **7**). The effective removal of pepsin throughout the purification steps was monitored by Western blots using purified goat anti-pepsin antibodies and quantitated using the ELISA method.

The results of the reverse phase HPLC analysis are shown in Table 1. In the first run, pepsin was detected in both the anion exchange membrane regeneration pool at a concentration of 40 μg/ml and in the digested precursor antibody pool after the CPG-pepsin and filtration step at a concentration of 48.3 μg/ml. By adding the total concentration of pepsin in both pools it was determined that 13.4 g of pepsin leached from the CPG-pepsin column during the digestion step in the first run. The data also revealed that the amount of filtration area used to remove leached pepsin was not enough at the flow rates and pH used in the first run. Nevertheless, the membrane was able to remove 21% of the total amount of pepsin leached from the pepsin-CPG column. Since the digested precursor antibody pool contained 10.6 g of leached pepsin that was not removed by the membrane, the purification yields from the pepsin-CPG digestion step and the SPHP step were low; 77 and 53%, respectively. Also, pepsin was detected in the SPHP pool by Western blot analysis.

TABLE 1

	Pepsin concentration
RUN #1	
Pepsin digested Ab pool	48.3 μg/ml
Pepsin digested Ab pool volume	220 L
Total amount of pepsin Ab pool	10.6 g
Membrane regeneration pool	40.4 μg/ml
Membrane regeneration volume	70 L
Total amount of pepsin Membrane pool	2.8 g
RUN #2	
Pepsin digested Ab pool	0
Pepsin digested Ab pool volume	630 L
Total amount of pepsin Ab pool	0
Membrane regeneration pool	230 μg/ml
Membrane regeneration volume	10 L
Total amount of pepsin Membrane pool	2.3 g

After the final purification step (Phenyl sepharose), pepsin was not detected by ELISA (Table 2) or by Western blot analysis. In the second run, the filtration area of the anion exchange membrane was doubled from 11,000 cm^2 to 22,000 cm^2. Pepsin was detected only in the anion exchange regeneration pool at a concentration of 230 μg/ml. Pepsin was not detected in the digested precursor antibody pool, after the CPG-pepsin digestion and filtration steps. The total amount of pepsin leached by the CPG-pepsin resin was 2.3 g. This value is 17% of the total amount of leached pepsin detected during the first run. Pepsin was not detected by reverse phase, pepsin ELISA or Western blots through the remaining purification steps of the second run. As a result of completely removing pepsin from the digested precursor pool, the purification yields from the pepsin-CPG digestion step and the SPHP were improved to 97 and 90%, respectively.

TABLE 2

Sample	Pepsin Values (mean of 2 reps.) [μg/ml]
Abx pool	<.5, <.5
Q pool run 1	7.4
Q pool run 2	<.5, <.5
SPHP Pool run 1	<.5, <.5
SPHP Pool run 2	<.5, <.5
HIC pool run 1	<.5, <.5
HIC pool run 2	<.5, <.5
Form. product run 1	<.5, <.5
Form. product run 2	<.5, <.5
Placebo formulation	<.5, <.5

The results of these experiments demonstrate that the use of a positively charged membrane in line immediately after the immobilized pepsin digestion step was advantageous. When pepsin was not completely removed by the membrane from the digested precursor antibody pool, decreased yields of functional antibody were obtained. Without being bound to any one theory, this was probably the result of overdigestion by the remaining pepsin in the pool. Furthermore when pepsin is not completely removed by the positively

charged membrane it was detected in the SPHP pool by Western blots. In the second run, leached pepsin was completely removed by the membrane. As a result the recovery yields for the pepsin digestion step and the SPHP cation exchange steps improved. Introduction of the anion exchange membrane improved the anti-CD18 purification process in two fundamental ways. First yields were improved by effectively removing pepsin from the CPG digestion pool, preventing further digestion. Second the overall efficiency and reproducibility of the process was improved by removing pepsin and other negatively charged contaminants early in the process.

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                         SEQUENCE LISTING


(1) GENERAL INFORMATION:

   (iii) NUMBER OF SEQUENCES: 7


(2) INFORMATION FOR SEQ ID NO:1:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 241 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

 Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
   1               5                  10                  15

 Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly Tyr Thr Phe Thr
                  20                  25                  30

 Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro Gly Lys Gly Leu
                  35                  40                  45

 Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly Gly Thr Ser His
                  50                  55                  60

 Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
                  65                  70                  75

 Thr Ser Thr Ala Tyr Met Gln Met Asn Ser Leu Arg Ala Glu Asp
                  80                  85                  90

 Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
                  95                 100                 105

 Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val
                 110                 115                 120

 Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
                 125                 130                 135

 Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly
                 140                 145                 150

 Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
                 155                 160                 165

 Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
                 170                 175                 180

 Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
                 185                 190                 195

 Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
                 200                 205                 210

 His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
                 215                 220                 225

 Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
                 230                 235                 240

 Leu
 241


(2) INFORMATION FOR SEQ ID NO:2:
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    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 214 amino acids
         (B) TYPE: Amino Acid
         (D) TOPOLOGY: Linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1               5                  10                  15

Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Asn
                20                  25                  30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser
                50                  55                  60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile
                65                  70                  75

Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80                  85                  90

Gly Asn Thr Leu Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 100                 105

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
               110                 115                 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
               125                 130                 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
               140                 145                 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
               155                 160                 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
               170                 175                 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
               185                 190                 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
               200                 205                 210

Arg Gly Glu Cys
           214


(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 36 amino acids
         (B) TYPE: Amino Acid
         (D) TOPOLOGY: Linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu Gly Gly Arg Met Lys Gln Leu Glu Asp Lys Val Glu Glu Leu
 1               5                  10                  15

Leu Ser Lys Asn Tyr His Leu Glu Asn Glu Val Ala Arg Leu Lys
                20                  25                  30

Lys Leu Val Gly Glu Arg
                35  36


(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 7 amino acids
         (B) TYPE: Amino Acid
         (D) TOPOLOGY: Linear
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-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Leu Xaa Xaa Xaa Xaa Xaa Xaa
  1               5       7
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2143 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC      50

TCATTGCTGA GTTGTTATTT AAGCTTTGGA GATTATCGTC ACTGCAATGC     100

TTCGCAATAT GGCGCAAAAT GACCAACAGC GGTTGATTGA TCAGGTAGAG     150

GGGGCGCTGT ACGAGGTAAA GCCCGATGCC AGCATTCCTG ACGACGATAC     200

GGAGCTGCTG CGCGATTACG TAAAGAAGTT ATTGAAGCAT CCTCGTCAGT     250

AAAAAGTTAA TCTTTTCAAC AGCTGTCATA AAGTTGTCAC GGCCGAGACT     300

TATAGTCGCT TTGTTTTTAT TTTTTAATGT ATTTGTAACT AGAATTCGAG     350

CTCGCCGGGG ATCCTCTAGA GGTTGAGGTG ATTTTATGAA AAAGAATATC     400

GCATTTCTTC TTGCATCTAT GTTCGTTTTT TCTATTGCTA CAAACGCGTA     450

CGCTGATATC CAGATGACCC AGTCCCCGAG CTCCCTGTCC GCCTCTGTGG     500

GCGATAGGGT CACCATCACC TGTCGTGCCA GTCAGGACAT CAACAATTAT     550

CTGAACTGGT ATCAACAGAA ACCAGGAAAA GCTCCGAAAC TACTGATTTA     600

CTATACCTCC ACCCTCCACT CTGGAGTCCC TTCTCGCTTC TCTGGTTCTG     650

GTTCTGGGAC GGATTACACT CTGACCATCA GCAGTCTGCA ACCGGAGGAC     700

TTCGCAACTT ATTACTGTCA GCAAGGTAAT ACTCTGCCGC CGACGTTCGG     750

ACAGGGCACG AAGGTGGAGA TCAAACGAAC TGTGGCTGCA CCATCTGTCT     800

TCATCTTCCC GCCATCTGAT GAGCAGTTGA AATCTGGAAC TGCCTCTGTT     850

GTGTGCCTGC TGAATAACTT CTATCCCAGA GAGGCCAAAG TACAGTGGAA     900

GGTGGATAAC GCCCTCCAAT CGGGTAACTC CCAGGAGAGT GTCACAGAGC     950

AGGACAGCAA GGACAGCACC TACAGCCTCA GCAGCACCCT GACGCTGAGC    1000

AAAGCAGACT ACGAGAAACA CAAAGTCTAC GCCTGCGAAG TCACCCATCA    1050

GGGCCTGAGC TCGCCCGTCA CAAAGAGCTT CAACAGGGGA GAGTGTTAAG    1100

CTGATCCTCT ACGCCGGACG CATCGTGGCG CTAGTACGCA AGTTCACGTA    1150

AAAACGGTAT CTAGAGGTTG AGGTGATTTT ATGAAAAAGA ATATCGCATT    1200

TCTTCTTGCA TCTATGTTCG TTTTTTCTAT TGCTACAAAC GCGTACGCTG    1250

AGGTTCAGCT GGTGGAGTCT GGCGGTGGCC TGGTGCAGCC AGGGGGCTCA    1300

CTCCGTTTGT CCTGTGCAAC TTCTGGCTAC ACCTTTACCG AATACACTAT    1350

GCACTGGATG CGTCAGGCCC CGGGTAAGGG CCTGGAATGG GTTGCAGGGA    1400

TTAATCCTAA AAACGGTGGT ACCAGCCACA ACCAGAGGTT CATGGACCGT    1450

TTCACTATAA GCGTAGATAA ATCCACCAGT ACAGCCTACA TGCAAATGAA    1500

CAGCCTGCGT GCTGAGGACA CTGCCGTCTA TTATTGTGCT AGATGGCGAG    1550
```

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```
GCCTGAACTA CGGCTTTGAC GTCCGTTATT TTGACGTCTG GGGTCAAGGA        1600

ACCCTGGTCA CCGTCTCCTC GGCCTCCACC AAGGGCCCAT CGGTCTTCCC        1650

CCTGGCACCC TCCTCCAAGA GCACCTCTGG GGGCACAGCG GCCCTGGGCT        1700

GCCTGGTCAA GGACTACTTC CCCGAACCGG TGACGGTGTC GTGGAACTCA        1750

GGCGCCCTGA CCAGCGGCGT GCACACCTTC CCGGCTGTCC TACAGTCCTC        1800

AGGACTCTAC TCCCTCAGCA GCGTGGTGAC CGTGCCCTCC AGCAGCTTGG        1850

GCACCCAGAC CTACATCTGC AACGTGAATC ACAAGCCCAG CAACACCAAG        1900

GTCGACAAGA AAGTTGAGCC CAAATCTTGT GACAAAACTC ACACATGCCC        1950

GCCGTGCCCA GCACCAGAAC TGCTGGGCGG CCGCATGAAA CAGCTAGAGG        2000

ACAAGGTCGA AGAGCTACTC TCCAAGAACT ACCACCTAGA GAATGAAGTG        2050

GCAAGACTCA AAAAGCTTGT CGGGGAGCGC TAAGCATGCG ACGGCCCTAG        2100

AGTCCCTAAC GCTCGGTTGC CGCCGGGCGT TTTTTATTGT TAA               2143
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 237 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe
-23         -20                 -15                 -10

Ser Ile Ala Thr Asn Ala Tyr Ala Asp Ile Gln Met Thr Gln Ser
             -5                   1               5

Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr
         10                  15                  20

Cys Arg Ala Ser Gln Asp Ile Asn Asn Tyr Leu Asn Trp Tyr Gln
         25                  30                  35

Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Tyr Thr Ser
         40                  45                  50

Thr Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser
         55                  60                  65

Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp
         70                  75                  80

Phe Ala Thr Tyr Tyr Cys Gln Gln Gly Asn Thr Leu Pro Pro Thr
         85                  90                  95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
        100                 105                 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser
        115                 120                 125

Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg
        130                 135                 140

Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
        145                 150                 155

Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr
        160                 165                 170

Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
        175                 180                 185

Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser
        190                 195                 200
```

-continued

```
Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
        205                 210             214


(2) INFORMATION FOR SEQ ID NO:7:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 300 amino acids
          (B) TYPE: Amino Acid
          (D) TOPOLOGY: Linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met Phe Val Phe
-23         -20                 -15                 -10

Ser Ile Ala Thr Asn Ala Tyr Ala Glu Val Gln Leu Val Glu Ser
            -5                   1                   5

Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys
         10                  15                  20

Ala Thr Ser Gly Tyr Thr Phe Thr Glu Tyr Thr Met His Trp Met
         25                  30                  35

Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Asn
         40                  45                  50

Pro Lys Asn Gly Gly Thr Ser His Asn Gln Arg Phe Met Asp Arg
         55                  60                  65

Phe Thr Ile Ser Val Asp Lys Ser Thr Ser Thr Ala Tyr Met Gln
         70                  75                  80

Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala
         85                  90                  95

Arg Trp Arg Gly Leu Asn Tyr Gly Phe Asp Val Arg Tyr Phe Asp
        100                 105                 110

Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr
        115                 120                 125

Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr
        130                 135                 140

Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe
        145                 150                 155

Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
        160                 165                 170

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr
        175                 180                 185

Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr
        190                 195                 200

Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys
        205                 210                 215

Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr
        220                 225                 230

Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Arg Met Lys
        235                 240                 245

Gln Leu Glu Asp Lys Val Glu Glu Leu Leu Ser Lys Asn Tyr His
        250                 255                 260

Leu Glu Asn Glu Val Ala Arg Leu Lys Lys Leu Val Gly Glu Arg
        265                 270                 275     277
```

We claim:

1. A method for recovering a polypeptide comprising:

(a) exposing a composition comprising a polypeptide to a reagent which binds to, or modifies, the polypeptide, wherein the reagent is immobilized on a solid phase; and then

(b) passing an effluent comprising the polypeptide eluted from or modified by the immobilized reagent, and any reagent leached from the solid phase, through a filter beating a charge which is opposite to the charge of the reagent in and at the pH of, the composition, so as to remove leached reagent from the effluent.

2. The method of claim **1** wherein the charge characteristics of the polypeptide in the composition in step (b) are such that the polypeptide passes through the filter.

3. The method of claim **1** wherein the filter is positively charged.

4. The method of claim **1** wherein the filter is negatively charged.

5. The method of claim **1** wherein the effluent is passed directly in line through the filter.

6. The method of claim **1** wherein the immobilized reagent is a protease.

7. The method of claim **6** wherein the protease is pepsin.

8. The method of claim **6** wherein the polypeptide exposed to the protease in step (a) is a precursor polypeptide and the protease removes a precursor domain from the polypeptide.

9. The method of claim **8** wherein the precursor domain comprises a leucine zipper.

10. The method of claim **9** wherein the polypeptide is an antibody.

11. The method of claim **10** wherein the antibody is a $F(ab')_2$ fragment.

12. The method of claim **10** wherein the antibody binds CD18.

13. The method of claim **9** wherein the leucine zipper is a yeast GCN4 leucine zipper.

14. The method of claim **9**, wherein the precursor polypeptide is an anti-CD18 antibody having the amino acid sequence of SEQ ID NO. 1 for the heavy chain, and SEQ ID NO 2 for the light chain.

15. The method of claim **1**, wherein the solid phase comprises controlled pore glass beads.

16. A method for recovering a polypeptide comprising removing a leached reagent from a composition comprising the polypeptide and the leached reagent by passing the composition through a filter bearing a charge opposite to that of the leached reagent at the pH of the composition, wherein the leached reagent was previously immobilized on a solid phase.

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