CURRENT PROTOCOLS IN MOLECULAR BIOLOGY

VOLUME 1

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SUPPLEMENT 38

A brief listing of new topics in Supplement 38 is provided below. Please see next page for a full listing of contents and instructions for inserting pages.

Also included are a supplement index, *The Red Book Bulletin,* and a pink Reader Response Card.

FULL CONTENTS OF SUPPLEMENT 38

Instructions for Adding Pages to Core Manual

Full contents for Supplement 38 are listed below, with instructions for removing old pages and inserting new pages. Every page included with Supplement 38 is labeled as such beneath the page number.

Remove pages	Insert pages	Subject	Primary change/notes
VOLUME 1			
vii-x	vii-x	Main table of contents	Reflects new *UNITS 10.21 & 16.21*, and revised *UNITS 16.9, 16.10, & 16.11*
VOLUME 2			
10.0.1-10.0.4	Chap. 10: 1-5	Chapter 10 contents	Reflects new *UNIT 10.21*
10.0.5-10.0.23	10.0.1-10.0.19	Chapter 10 introduction	Revision
——	10.21.1-10.21.27	Overview of mass spectrometry	New *UNIT 10.21*
16.0.1-16.0.4	Chap. 16: 1-4	Chapter 16 contents	Reflects revised *UNITS 16.9, 16.10, & 16.11*, and new*16.21*
16.0.5-16.0.6	16.0.1-16.0.3	Chapter 16 introduction	Revision
16.9.1-16.9.6	16.9.1-16.9.10	Overview of baculovirus expression system	Revised *UNIT 16.9*
16.10.1-16.10.8	16.10.1-16.10.17	Maintenance of insect cell cultures and generation of recombinant baculoviruses	Revised *UNIT 16.10*
16.11.1-16.13.7 (32 pages)	16.11.1-16.13.7 (25 pages)	Purification of proteins using baculovirus	Revised *UNIT 16.11*
——	16.21.1-16.21.9	Inducible gene expression	New *UNIT 16.21*
VOLUME 3			
A.1B.1-A.1C.1 (2 pages)	A.1B.1-A.1C.1 (2 pages)	Useful measurements and data	Revision
A.1C.8-A.1C.10	A.1C.8-A.1C.12	Characteristics of amino acids	New tables in *APPENDIX 1C*
Supp. 37 index (2 pages)	Supp. 37 & 38 index (3 pages)		New entries covering Supplements 37 & 38

Corrections

The following errors have been detected since publication of the last supplement. NOTE: These changes are primarily scientific, not typographical, errors; thus it is crucial that they be entered in your manual for optimal use of the protocols.

Page	Error	Correction
10.18.4, Additional materials, line 2	"150-mm^2 flask"	"150-**cm^2** flask"
11.2.19, Reagents and solutions, MUP and NPP substrate solutions, line 2	"$NaCO_3$"	"Na_2CO_3"
14.5.4, Reagents and solutions	"Gelvatol"	"Gelvatol **(now called Airvol, from Air Products and Chemicals)**"
15.5.13, step 28	"final DNA concentration is 1 µg/ml"	"final DNA concentration is 1 µg/**µl**"
15.5.19, Vent DNA polymerase mix, line 2	"Add 0.5 µl (10 U)"	"Add 0.5 µl (**1 U**)"

Contents, Volumes 1, 2, and 3

4 Preparation and Analysis of RNA

5 Construction of Recombinant DNA Libraries

6 Screening of Recombinant DNA Libraries

11 Immunology

12 DNA-Protein Interactions

13 *Saccharomyces cerevisiae*

14 In situ Hybridization and Immunohistochemistry

15 The Polymerase Chain Reaction

16 Protein Expression

17 Preparation and Analysis of Glycoconjugates

Appendices

Index

FOREWORD

The breadth of knowledge required for modern research in molecular biology is truly staggering. A single series of experiments can encompass genetic manipulation of specific bacterial strains and their phage, the appropriate use of several highly specific enzymes, and the culturing and genetic engineering of mammalian cells. For individuals who have followed the evolution of research in molecular biology, it was surprising to watch the mammalian λ immunoglobulin gene being cloned in the prokaryotic λ phage. Neither immunologists nor bacterial geneticists expected the ultimate union when the phage and gene were named. Now a single individual must master both fields to be successful. Research that depends upon such breadth of knowledge needs protocol books that explain why certain methods are used and provide references for further reading. This manual fulfills that need.

All scientists realize that they figuratively stand on the shoulders of their predecessors. Nothing more concretely reflects this dependence than a laboratory protocol book. It is a monument to the hours of labor that students, technicians and other professionals have expended in developing methods that work every time. Good methods have another characteristic: they have been honed to the minimum of time and effort required to achieve the desired results. Most investigators collect personal protocol books of "tried and true" recipes. This new manual with its continuously growing and evolving set of protocols is an excellent basis from which to begin such a collection.

There are two unique features of this manual that are in tune with the rapid advances in molecular biology. First, the manual evolves continuously with the addition of new protocols in emerging areas of research. These new protocols are added as quarterly supplements to the core manual. Second, the manual evolves through a "network" of the users. Readers are encouraged to contribute corrections and improvements on techniques in each supplement. Thus, the book is designed to grow and change as biology grows and changes.

Molecular biology has always been an intensely experimental science. Young people, more commonly, have the energy and intensity to push the field forward. The excellent group of young scientists who have contributed to this book are representatives of this tradition.

Phillip A. Sharp
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February 1993

PREFACE

Although mastery of the techniques in this manual will enable the reader to pursue research in molecular genetics, the manual is not intended to be a substitute for graduate-level courses in molecular biology or a comprehensive textbook in the field. Introductory texts that we recommend include: *Molecular Biology of the Gene* (4th ed.), by J.D. Watson, N.H. Hopkins, J.W. Roberts, J.A. Steitz, and A.M. Weiner; *Molecular Genetics: An Introductory Narrative,* by G.S. Stent and R. Calendar; *From Genes to Clones,* by E.-L. Winnacker; and *Genetics and Molecular Biology,* by R. Schleif. In addition, *An Introduction to Genetic Analysis,* by D.T. Suzuki, A.J.F. Griffiths, J.H. Miller, and R.C. Lewontin, is a good place to learn classical genetics, and *The Molecular Biology of the Cell,* by B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts, and J.D. Watson is a compendium of useful information about many aspects of cellular and molecular biology. Finally, we strongly recommend that readers gain first-hand experience in basic techniques and safety procedures by working in a molecular biology laboratory.

An inevitable hazard of manual writing is that protocols become obsolete or that new techniques are developed—usually just as the pages are going to press. This problem is particularly severe in a fast-moving field such as molecular genetics. To safeguard our manual from inexorable obsolescence, and as a means of correcting errors in a timely manner, we provide a quarterly updating service. The looseleaf format will facilitate the replacement of pages that contain errors or have become obsolete and will accommodate the addition of new protocols to the appropriate section of the manual. The publisher can provide further details about subscribing to the quarterly supplements.

HOW TO USE THIS MANUAL

Format and Organization

This publication is available in both looseleaf and CD-ROM format. The material covered in the two versions is identical.

For looseleaf purchasers, two binders are provided to accommodate the growth of the manual via the quarterly update service. The first volume contains Chapters 1 through 9, and the second volume holds Chapter 10 through the appendices, including any new chapters to be added with the quarterly supplements. A full index and table of contents are included with both volumes. The looseleaf format of the binders allows easy insertion of new pages, units, and chapters that are added. The index and table of contents are updated with each supplement.

CD-ROM purchasers receive a completely new disc containing the entire manual every quarter. The CD-ROM User's Guide describes in detail the many features designed for accessing information presented in the CD. Topics of interest can be located primarily by using either the search index (by typing in the desired word or string of words) or by scanning through the expandable table of contents and selecting the desired units. In addition, hyperlinks jump between related units when cross-references are selected and from solutions and reagents listed in the materials list to their corresponding recipes.

Subjects in this manual are organized by chapters and sections, and protocols are contained in units. Units generally describe a method and include one or more protocols with listings of materials, the protocol steps and annotations, recipes for unique reagents and solutions, and commentaries on the "hows" and "whys" of the method. Overview units contain theoretical discussions that lay the foundation for subsequent protocols. Page numbering in the looseleaf version reflects this modular arrangement; for example, page

7.4.2 refers to Chapter 7 (DNA sequencing), Unit 4 (the dideoxy method), page 2 of that particular unit.

Many reagents and procedures are employed repeatedly throughout the manual. Rather than duplicate this information, cross-references among units are used extensively. Early chapters (and appendices) describe these commonly used techniques (basic microbiology and basic manipulations of enzymes, DNA, and RNA), while later chapters describe their application (constructing libraries, DNA sequencing, mutagenesis, transfection, and protein analysis). Thus, whenever a particular enzyme is used in a protocol, the appropriate unit in Chapter 3—describing reaction conditions for that enzyme—is cross-referenced (e.g., *UNIT 3.7* for reverse transcriptase). Similarly, throughout the book readers are referred to *UNIT 1.3* for spreading or streaking a plate, to *UNIT 2.1* for phenol extraction/alcohol precipitation, to *UNIT 2.5* for agarose gel electrophoresis, and so on. By turning to these units, the reader will find instructions for the techniques, recipes for relevant reagents, and commentary. As a result, protocols in the later chapters of the book—which can be lengthy and complex—are not overburdened with steps describing auxiliary procedures required to prepare, purify, and analyze the sample or molecule of interest.

Introductory and Explanatory Information

Because this publication is first and foremost a compilation of molecular biology techniques we have not provided extensive instructive material. We have, however, included explanatory information where required to help readers gain an intuitive grasp of the procedures. First, the chapter, section, and unit introductions describe how the protocols that follow connect to each other. Second, annotations to the actual protocol steps describe what is happening. Third, the commentary sections that close each unit give background information and review the relevant literature so that readers are aware of the historical and theoretical development of a technique, as well as alternative approaches. Fourth, key references are sometimes included to inform users of particularly useful background reading or original descriptions or applications of the technique. Finally, in some cases special "overview" units have been included early in the chapter.

Protocols

Many units in the manual contain groups of protocols. The *basic* protocol is presented first in each unit and is generally the recommended approach. *Alternate* protocols are provided where different equipment or reagents can be employed to achieve similar ends, where the starting material requires a variation in approach, or where requirements for the end product differ from those in the basic protocol. *Support* protocols describe additional steps that are required to perform the basic or alternate protocols; these steps are separated from the core protocol because they might be applicable to other uses in the manual, or because they are performed in a time frame separately from the basic protocol steps.

Reagents and Solutions

Reagents required for a protocol are listed in the "materials" list before the procedure begins. Many are common stock solutions, others are commonly used buffers or media, while others are solutions unique to a particular protocol. Recipes for the latter solutions are provided in each unit, following the protocols (and before the commentary) under the heading Reagents and Solutions. It is important to note that the *names* of some of these special solutions might be similar from unit to unit (e.g., hybridization solution, high-salt solution, etc.) while the *recipes* differ; thus, make certain that reagents are prepared from the proper recipes. On the other hand, recipes for commonly used buffers and media are provided once in an appendix or in early units in the manual. These universal recipes are

cross-referenced parenthetically in the materials lists rather than repeated with every usage.

NOTE: *Deionized, distilled water should be used in all protocols in this manual, and in the preparation of all reagents and solutions.*

Commercial Suppliers

In some instances throughout the manual, we have recommended commercial suppliers of chemicals, biological materials, or equipment. This has been avoided wherever possible, because preference for a specific brand is subjective and is generally not based on extensive comparison testing. Our guidelines for recommending a supplier are that (1) the particular brand has actually been found to be of superior quality, or (2) the item is difficult to find in the marketplace. An appendix lists the full names and addresses of all recommended suppliers, but these are by no means the only vendors of biological supplies. Readers may experiment with substituting their own favorite brands.

Safety Considerations

Anyone carrying out these protocols will encounter the following hazardous or potentially hazardous materials: (1) radioactive substances, (2) toxic chemicals and carcinogenic or teratogenic reagents, (3) pathogenic and infectious biological agents, and (4) recombinant DNA. Most governments regulate the use of these materials; it is essential that they be used in strict accordance with local and national regulations. Cautionary notes are included in many instances throughout the manual, but we emphasize that users must proceed with the prudence and precaution associated with good laboratory practice, and that all materials be used in strict accordance with local and national regulations.

Reader Response

Most of the protocols included in this manual are used routinely in our own laboratories. These protocols work for us; to make them work for you we have annotated critical steps and included critical parameters and troubleshooting guides in the commentaries to most units. However, the successful evolution of this manual depends upon readers' observations and suggestions. Consequently, a self-mailing, reader-response survey is included at the back of the manual (and is included with each supplement); we encourage readers to send in their comments.

ACKNOWLEDGMENTS

Continuing to keep this manual up to date would be impossible without massive assistance from the Current Protocols staff at John Wiley & Sons. Among those who help us, we are extremely grateful to Kathy Wisch, Janet Blair, Hazel Chan, Rebecca Barr, and Elizabeth Konkle. We are particularly indebted to Sarah Greene and her staff at Greene Publishing Associates who initially conceived of this project and helped to shape it with skill and unbounded patience.

We are especially grateful to our co-workers who have helped with the manual by contributing material to it, by commenting on the chapters, or by field-testing the procedures. To those people—in our own labs in Boston, and in academic and industrial labs all over the world—we offer our deepest thanks.

Frederick M. Ausubel, Roger Brent, Robert E. Kingston, David D. Moore, J. G. Seidman, John A. Smith, and Kevin Struhl

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ESCHERICHIA COLI, PLASMIDS, AND BACTERIOPHAGES

1

INTRODUCTION

Mastery of current DNA technology requires familiarity with a small number of basic concepts and techniques. The goal of this chapter is to present this information concisely, yet in enough detail to be useful when a procedure goes wrong. Section I is devoted to *Escherichia coli*. Recipes are provided for media that support *E. coli* growth, as well as instructions for making the simple tools needed to work with bacterial cells. Growth of *E. coli* in liquid and solid media is then detailed. The final unit in Section I describes a few detailed aspects of *E. coli* biology learned from classical bacterial genetic studies, the understanding of which is especially relevant to the techniques used in modern DNA work.

The remainder of the chapter discusses vectors used to introduce foreign DNA into *E. coli*. For the purposes of this chapter, vectors are said to be derived from plasmids, from bacteriophage lambda and related phages, or from filamentous phages. (Many modern vectors incorporate elements from more than one of these classes, and it is likely that this classification scheme will be hopelessly outdated by the time this chapter is revised.) Section II is concerned with plasmid vectors. Following a brief introduction to plasmid biology, procedures are described for purifying small and large amounts of plasmid DNA ("minipreps" and large preps). Finally, procedures for reintroducing plasmid DNA into bacterial cells are described. Section III covers vectors derived from bacteriophages. The biology of bacteriophage lambda is first introduced, followed by detailed aspects of biology that are especially significant when lambda derivatives are used as cloning vectors. Protocols in this section describe techniques for manipulating lambda-derived vectors, making single plaques, making and titering phage stocks, and isolating phage DNA. Finally, Section IV covers the biology and manipulation of vectors derived from filamentous phages.

This chapter will be meaningful primarily to readers with some knowledge of the principles of molecular biology. Several books on molecular biology are recommended in the preface. For further advanced reading in the topics of this chapter, we recommend five books, all from Cold Spring Harbor Laboratory: *Methods in Molecular Genetics* (Miller, 1972), *Advanced Bacterial Genetics* (Davis et al., 1980), *The Bacteriophage Lambda* (Hershey, 1971), *Lambda II* (Hendrix et al., 1983), and *Experiments with Gene Fusions* (Silhavy et al., 1984).

Many terms and jargon used by molecular biologists are introduced in this chapter. These terms are italicized at their first mention, and are defined below.

alpha fragment peptide containing the amino terminus of β-galactosidase, the *lacZ* gene product. Alpha fragments lack enzymatic activity, but can associate with omega fragments (see below) to form proteins whose β-galactosidase activity has been restored.

alpha-complementation restoration of β-galactosidase activity to omega fragments by association with alpha fragments.

amplification increase in copy number of some plasmids that occurs when host protein synthesis is inhibited.

cloning site site on a vector into which foreign DNA is inserted.

competent state in which bacterial or yeast cells are able to take up foreign DNA (for example, as the result of calcium treatment).

cos site of action of phage lambda *ter* function. Cos site is cut by *ter* to yield two cohesive ends (cos ends).

dilution, 10^x-fold a solution or suspension that contains $1/10^x$ as much (10^{-x} as much) of the dissolved or suspended species as does the

starting liquid. For example, to "do a 10^2-fold dilution" is to dilute a solution 100×.

early-log phase period during the growth of a culture after the lag period. During early log phase growth, cells have begun exponential growth.

efficiency of plating (EOP) titer of bacterial colonies or phage plaques under some experimental conditions divided by the titer of bacteria or phage obtained by growth on some reference medium.

exponential growth period during which the number of cells in the culture increases as an exponential function of time, that is, during which cell number = ke^t.

F factor genetic element found in some strains of *E. coli* and related species. F encodes proteins used in formation of sex pili which allow its transfer from bacterium to bacterium.

female strain strain that does not contain the F factor and that receives genetic information when crossed with a strain containing F.

helper phages bacteriophages that encode essential proteins and that allow other phages which do not encode these essential proteins to grow.

incompatible phenomenon in which two plasmids cannot replicate in the same cell without continual selection for both of them.

incompatibility group consists of plasmids that cannot be maintained together in the same cell. Compatible plasmids belong to different incompatibility groups.

induction the onset of transcription of a new gene or operon, usually in response to some environmental stimulus. Phage induction or lysogenic induction describe the process in which prophage excise from the chromosome of bacteria that harbor them and begin to grow lytically.

inoculation introduction of cells into a container of sterile growth medium.

lag period period just after inoculation of a culture when cells have not yet begun to grow exponentially.

late-log phase last period of exponential growth of a culture, after which growth slows and then stops altogether due to nutrient exhaustion or accumulation of waste products.

lawn uniform layer of bacteria that covers the surface of a plate.

log phase period during growth of a culture in which cells are growing exponentially.

low-copy-number plasmids plasmids found in less than about 20 copies per cell when cells containing them are grown in rich medium.

lysogen *E. coli* cell or strain that harbors a dormant bacteriophage.

male strain strain of bacteria that contains the F factor.

male-specific phages bacteriophages that only grow on male strains because they adsorb to sex pili.

marker detectable genetic difference between one organism and another (usually wild-type) organism of the same species.

minimal medium growth medium for cells that contains only salts, vitamins, trace elements, and simple compounds which serve as carbon, nitrogen, and phosphorous sources.

miniprep small-scale preparation or purification of some desired species, usually of plasmid or phage DNA.

mobilization transfer of DNA from one cell to another caused by a mobile genetic element such as the F factor.

multiplicity of infection (MOI) ratio of infecting bacteriophage to host cells.

nonsense suppression the insertion of amino acids into proteins at positions where translation would normally not occur because the mRNA contains a UAG (amber), UAA (ochre) or UGA nonsense codon.

nonsense suppressor tRNA that inserts amino acids at nonsense codons. The term is sometimes used for the genes encoding these tRNAs.

omega fragment protein containing the carboxy-terminal fragment of β-galactosidase. This protein lacks enzymatic activity, but β-galactosidase activity can be restored when the peptide is complexed with an alpha fragment.

ori (origin) site on genome at which DNA replication begins.

outgrowth the growth of freshly transformed cells under nonselective conditions for enough time to allow proteins encoded by the foreign DNA to be expressed.

overnight a small, freshly saturated liquid culture of bacteria.

packaging extract extract from special strains of *E. coli* that contains bacteriophage lambda head proteins, tail proteins, and packaging proteins. Phage DNA added to such an extract is assembled into phage particles.

par site on some plasmids which ensures that each daughter cell receives a plasmid copy.

pilot protein protein in the coat of filamentous phages that helps phage DNA enter the cell.

plates petri dishes filled with solid medium, used to grow separated bacterial colonies or plaques. The term is sometimes used to refer to 96-well microtiter dishes.

plating out the placement of bacteria or phage on plates so that colonies or plaques are formed.

polylinker stretch of DNA that contains contiguous restriction sites.

prophage dormant bacteriophage, usually integrated into the host chromosome, that replicates with the host bacterium.

relaxed control applies to plasmids whose replication does not depend on the bacterial cell cycle.

replicative form double-stranded circular filamentous phage DNA found inside infected cells.

replicator stretch of DNA on a phage or plasmid that enables the phage or plasmid to replicate.

rich medium growth medium that contains complex organic molecules (peptides, nucleotides, etc.). Typical components of rich media include tryptone (made from beef) and yeast extract (made from yeast).

rolling-circle replication mechanism of replication sometimes used by circular molecules in which DNA polymerase continually circumnavigates the template, and thus synthesizes a long tail.

satellite colonies small colonies that grow around a large colony on a plate containing selective medium. These are usually composed of cells unable to grow on selective medium, but which are able to grow near the large colony because the cells in the large colony neutralize the selective agent.

saturated culture culture of cells in liquid medium that has stopped growing because nutrients are exhausted or because waste products have accumulated.

SOS response response of *E. coli* to DNA damage or other treatments that inhibit DNA replication. Lambda-derived phages are induced during this response.

stringent control applies to plasmids whose replication is synchronized with the *E. coli* cell cycle.

temperate describes bacteriophages capable of lysogenic growth.

transfection introduction of bacteriophage DNA into competent *E. coli* cells. Also describes the introduction of any DNA (including plasmid DNA) into cells of higher eukaryotes.

transformation introduction of plasmid DNA into *E. coli* or yeast. Also used to denote any of a number of changes in cultured higher eukaryotic cells to characteristics more typical of cancer cells (immortal growth, loss of contact inhibition, etc.).

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SECTION I

ESCHERICHIA COLI

Escherichia coli is a rod-shaped bacterium with a circular chromosome about 3 million base pairs (bp) long. It can grow rapidly on *minimal medium* that contains a carbon compound such as glucose (which serves both as a carbon source and an energy source) and salts which supply nitrogen, phosphorus, and trace metals. *E. coli* grows more rapidly, however, on a *rich medium* that provides the cells with amino acids, nucleotide precursors, vitamins, and other metabolites that the cell would otherwise have to synthesize. The purpose of this first section is to provide basic information necessary to grow *E. coli*. A more detailed introduction to certain aspects of *E. coli* biology may be found in *UNIT 1.4*.

When *E. coli* is grown in liquid culture, a small number of cells is first *inoculated* into a container of sterile medium. After a period of time, called the *lag period*, the bacteria begin to divide. In rich medium a culture of a typical strain will double in number every 20 or 30 min. This phase of *exponential growth* of the cells in the culture is called *log phase* (sometimes subdivided into *early-log*, *middle-log*, and *late-log phases*). Eventually the cell density increases to a point at which nutrients or oxygen become depleted from the medium, or at which waste products (such as acids) from the cells have built up to a concentration that inhibits rapid growth. At this point, which, under normal laboratory conditions, occurs when the culture reaches a density of 1 to 2×10^9 cells/ml, the cells stop dividing rapidly. This phase is called *saturation* and a culture that has just reached this density is said to be freshly saturated.

With very few exceptions, bacterial strains used in recombinant DNA work are derivatives of *E. coli* strain K-12. Most advances in molecular biology until the end of the 1960s came from studies of this organism and of bacteriophages and plasmids that use it as a host. Much of the cloning technology in current use exploits facts learned during this period.

UNIT 1.1

Media Preparation and Bacteriological Tools

Recipes are provided below for minimal liquid media, rich liquid media, solid media, top agar, and stab agar. Tryptone, yeast extract, agar (Bacto-agar), nutrient broth, and Casamino Acids are from Difco. NZ Amine A is from Hunko Sheffield (Kraft).

MINIMAL MEDIA

Ingredients for these media should be added to water in a 2-liter flask and heated with stirring until dissolved. The media should then be poured into separate bottles with loosened caps and autoclaved at 15 lb/in^2 for 15 min. Do not add nutritional supplements or antibiotics to any media until it has cooled to <50°C. After the bottles cool to below 40°C, the caps can be tightened and the concentrated media stored indefinitely at room temperature.

5× M9 medium, per liter

30 g Na_2HPO_4
15 g KH_2PO_4
5 g NH_4Cl
2.5 g NaCl
15 mg $CaCl_2$ (optional)

5× M63 medium, per liter

10 g $(NH_4)_2SO_4$
68 g KH_2PO_4
2.5 mg $FeSO_4 \cdot 7H_2O$
Adjust to pH 7 with KOH

5× A medium, *per liter*

5 g $(NH_4)_2SO_4$
22.5 g KH_2PO_4
52.5 g K_2HPO_4
2.5 g sodium citrate·$2H_2O$

Before they are used, concentrated media should be diluted to 1× with sterile water and the following sterile solutions, per liter:

1 ml 1 M $MgSO_4 \cdot 7H_2O$
10 ml 20% carbon source (sugar or glycerol)
and, if required:
0.1 ml 0.5% vitamin B1 (thiamine)
5 ml 20% Casamino Acids *or*
L amino acids to 40 µg/ml *or*
DL amino acids to 80 µg/ml
Antibiotic (see Table 1.4.1)

RICH MEDIA

Unless otherwise specified, rich media should be autoclaved for 25 min. Antibiotics and nutritional supplements should be added only after the solution has cooled to 50°C or below. A flask containing liquid at 50°C feels hot but can be held continuously in one's bare hands.

H medium, *per liter*

10 g tryptone
8 g NaCl

Lambda broth, *per liter*

10 g tryptone
2.5 g NaCl

LB medium, *per liter*

10 g tryptone
5 g yeast extract
5 g NaCl
1 ml 1 N NaOH

The original recipe for LB medium (sometimes referred to as Luria or Lenox broth), does not contain NaOH. There are many different recipes for LB that differ only in the amount of NaOH added. We use this formula in our own work. Even though the pH is adjusted to near 7 with NaOH, the medium is not very highly buffered, and the pH of a culture growing in it drops as it nears saturation.

NZC broth, *per liter*

10 g NZ Amine A
5 g NaCl
2 g $MgCl_2 \cdot 6H_2O$
Autoclave 30 min
5 ml 20% Casamino Acids

Superbroth, *per liter*

32 g tryptone
20 g yeast extract
5 g NaCl
5 ml 1 N NaOH

TB (terrific broth)

12 g Bacto tryptone
24 g Bacto yeast extract
4 ml glycerol

Add H_2O to 900 ml and autoclave, then add to above sterile solution 100 ml of a sterile solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4.

Tryptone broth, *per liter*

10 g tryptone
5 g NaCl

2× TY medium, *per liter*

16 g tryptone
10 g yeast extract
5 g NaCl

TYGPN medium, *per liter*

20 g tryptone
10 g yeast extract
10 ml 80% glycerol
5 g Na_2HPO_4
10 g KNO_3

SOLID MEDIA

Liquid media can be solidified with agar. For minimal plates, dissolve the agar in water and autoclave separately from the minimal medium; autoclaving the two together will give rise to an insoluble precipitate. For rich plates, autoclave the agar together with the other ingredients of the medium. Cool the agar to about 50°C and add other ingredients if necessary. At this temperature, the medium will stay liquid indefinitely, but it will rapidly solidify if its temperature falls much below 45°C. Finally, pour the medium into sterile disposable petri dishes (*plates*) and allow to solidify.

Freshly poured plates are wet and unable to absorb liquid spread onto them. Moreover, plates that are even slightly wet tend to exude moisture underneath bacteria streaked on them, which can cause the freshly streaked bacteria to float away. So for most applications, dry the plates by leaving them out at room temperature for 2 or 3 days, or by leaving them with the lids off for 30 min in a 37°C incubator or in a laminar flow hood. Store dry plates at 4°C, wrapped in the original bags used to package the empty plates.

Minimal Plates

Autoclave 15 g agar in 800 ml water for 15 min. Add sterile concentrated minimal medium and carbon source. After medium has cooled to about 50°C, add supplements and antibiotics. Pouring 32 to 40 ml medium into each plate, expect about 25 to 30 plates per liter.

Rich Plates

To ingredients listed below, add water to 1 liter and autoclave 25 min. Pour LB and H plates with 32 to 40 ml medium, in order to get 25 to 30 plates per liter. Pour lambda plates with about 45 ml medium for about 20 plates per liter.

H plates, *per liter*

10 g tryptone
8 g NaCl
15 g agar

Lambda plates, *per liter*

10 g tryptone
2.5 g NaCl
10 g agar

LB plates, *per liter*

10 g tryptone
5 g yeast extract
5 g NaCl
1 ml 1 N NaOH
15 g agar or agarose

Additives

Antibiotics (if required):
Ampicillin to 50 µg/ml
Tetracycline to 12 µg/ml
Other antibiotics, see Table 1.4.1

Galactosides (if required):
Xgal to 20 µg/ml
IPTG to 0.1 mM
Other galactosides, see Table 1.4.2

TOP AGAR

Top agar is used to distribute phage or cells evenly in a thin layer over the surface of a plate. In a typical application, molten top agar is mixed with bacteria and the mixture poured onto a plate to make a thin layer that is allowed to solidify. This layer of cells then grows denser, forming the opaque *lawn* of cells. Top agar contains less agar than plates, and so stays molten for days when it is kept at 45° to 50°C. Top agarose is sometimes used when DNA is to be prepared directly from phage, and is also used when libraries are plated out to be screened by plaque lifting (*UNIT 5.9*).

Prepare top agar in 1-liter batches, autoclave for 15 min to melt, cool to 50°C, swirl to mix, pour into separate 100-ml bottles, reautoclave, cool, and store at room temperature. Before use, melt the agar by heating in a water bath or microwave oven (see *UNIT 1.11*) then cool to and hold at 45° to 50°C.

H top agar, *per liter*

10 g tryptone
8 g NaCl
7 g agar

LB top agar, *per liter*

10 g tryptone
5 g yeast extract
5 g NaCl
7 g agar

Lambda top agar, *per liter*

10 g tryptone
2.5 g NaCl
7 g agar

Top agarose, *per liter*

10 g tryptone
8 g NaCl
6 g agarose

STAB AGAR

Stab agar is used for storing bacterial strains (see *UNIT 1.3*).

***Stab agar,** per liter*

10 g nutrient broth
5 g NaCl
6 g agar
10 mg cysteine·Cl
10 mg thymine

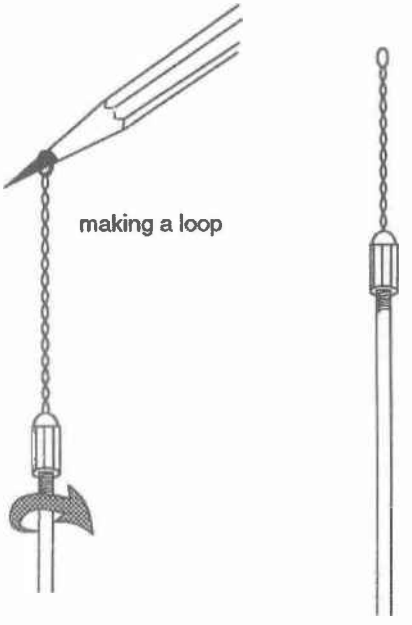
Thymine is included so that thy⁻ bacteria can grow. Cysteine is thought to increase the amount of time bacteria can survive in stabs.

TOOLS

Inoculating Loops

Inoculating loops are used to move small numbers of bacteria or phage to a plate or to a new container of liquid medium. Inoculating loops may be purchased from any general scientific supply company. However, most researchers prefer to use loops made in the laboratory. These are made by inserting both ends of a 10-in. piece of 28-G platinum wire into an inoculating loop holder (also widely available) and twirling the holder while tugging on the middle of the wire with the point of a pencil (see sketch 1.1A).

Sterilize the loop by holding it in a bunsen burner flame until it is red hot. Cool the loop by touching it to a sterile portion of the surface of an agar plate until it stops sizzling.

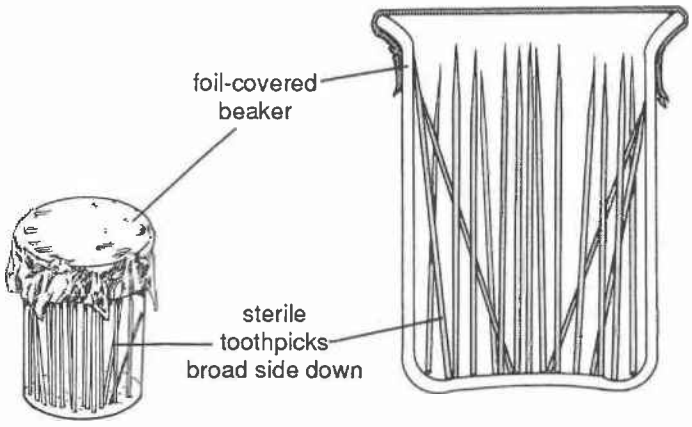


Sketch 1.1A

Sterile Toothpicks

The broad side of flat wooden toothpicks may also be used for streaking out bacteria. Round wooden toothpicks, or the pointed end of flat toothpicks, are sometimes used to pick individual colonies or phage plaques. To sterilize, place toothpicks in a small beaker, cover the beaker with foil, and autoclave. Alternatively, simply autoclave the whole box of toothpicks and hold them in the middle when picking them up out of the opened box. It is convenient to put used toothpicks into another smaller beaker which,

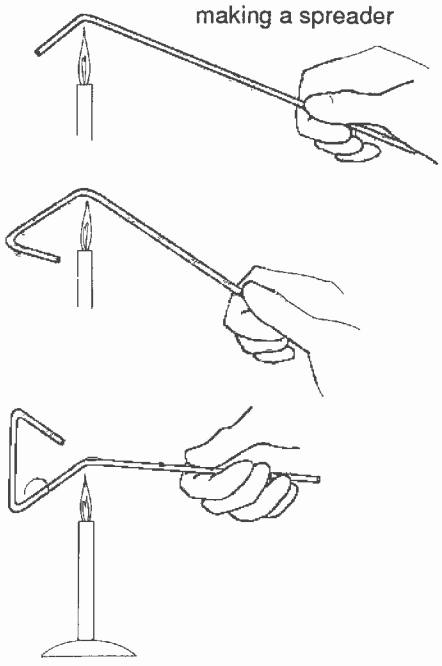
when full, is covered with foil and autoclaved. Used toothpicks can be saved, reautoclaved, and used again (see sketch 1.1B).



Sketch 1.1B

Spreaders

Spreaders are used to distribute liquid containing bacterial cells evenly over a plate. They are made by heating and bending a piece of 4-mm glass tubing (see sketch 1.1C). Less durable spreaders can be made from a Pasteur pipet. Before each use, sterilize the spreader by dipping the triangular part into a container of ethanol, passing the spreader through a gas flame to ignite the ethanol, and letting the flame burn out. Be careful not to ignite the ethanol in the container. Cool the spreader by touching it to the surface of an agar plate that has not yet been spread with cells.



Sketch 1.1C

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Growth in Liquid Media

BASIC PROTOCOL

GROWING AN OVERNIGHT CULTURE

Small freshly saturated cultures of *E. coli* are called *overnights*. To make an overnight, remove the cap from a sterile 16- or 18-mm culture tube. Working quickly to minimize contact of the tube with the possibly contaminated air, use a sterile pipet to transfer 5 ml of liquid medium into the tube. Inoculate the liquid with a single bacterial colony by touching a sterile inoculating loop to the colony, making certain that some of the cells have been transferred to the loop, and then dipping the loop into the liquid and shaking it a bit. Replace the tube's cap, and place the tube on a roller drum at 60 rpm, 37°C. Grow until the culture is freshly saturated (at a density of 1 to 2×10^9 cells/ml, which typically takes at least 6 hr).

GROWING LARGER CULTURES

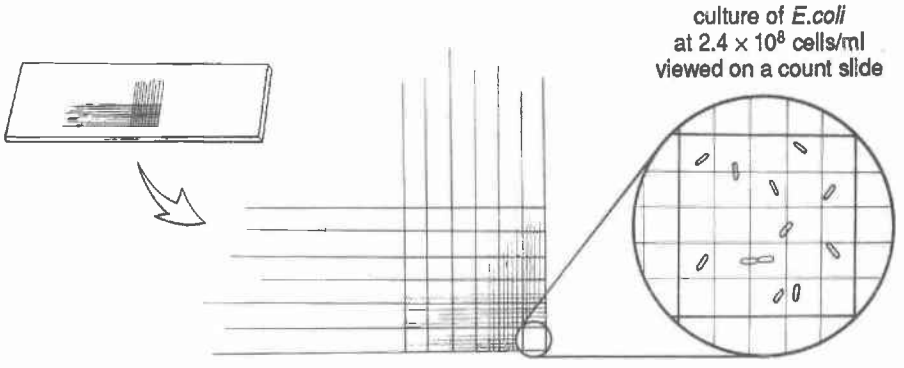
Larger cultures are generally inoculated with overnight cultures diluted 1:100. Use an Erlenmeyer or baffle flask whose volume is at least 5 times the volume of the culture. Grow the culture at 37°C with vigorous agitation (~300 rpm) to ensure proper aeration. If it is necessary to grow a culture without shaking (for example, if the strain is temperature-sensitive for growth and no low-temperature shaker is available), then, to ensure that the cells get adequate aeration, grow the culture in an Erlenmeyer flask whose volume is at least 20 times that of the culture.

BASIC PROTOCOL

MONITORING GROWTH

With a Count Slide

Take a clean count slide (or hemacytometer) and cover it with a clean cover slip. Dip a 0.1- or 1-ml pipet into the culture medium, allow a small drop of liquid to form on the end of the pipet, and touch it lightly to the surface of the slide at the periphery of the cover slip. The liquid will quickly spread under the cover slip. Put the slide on the stage of a phase-contrast microscope set to 400×, and focus on the cells. Each cell in a small square is equivalent to 2×10^7 cells/ml (see sketch 1.2A).



Sketch 1.2A

With a Spectrophotometer

The concentration of cells in a culture can also be determined with a spectrophotometer by measuring the amount of 600-nm light scattered by the culture. The level of absorbance (A) at 600 nm will depend on the distance between the cuvette and the detector and will vary among spectrophotometers, often by a factor of 2. It is thus wise

to calibrate each instrument by recording the OD_{600} (sometimes expressed as A_{600}) of a culture that contains a known number of cells determined by some other method, such as observation on a count slide or titering for viable colonies (*UNIT 1.3*).

If the culture is visibly turbid, also measure a *10-fold dilution* of it. For a culture grown in rich medium, a good rule of thumb is that each 0.1 OD unit is roughly equivalent to 10^8 cells/ml.

Calculate the number of cells/ml from whichever suspension (the undiluted or the diluted) has an OD_{600} <1.

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Growth on Solid Media

BASIC PROTOCOL

TITERING AND ISOLATING BACTERIAL COLONIES BY SERIAL DILUTIONS

Bacteria are grown from single colonies to ensure that each cell in a population is descended from a single founder cell, and thus to help ensure that each cell in the culture has the same genetic makeup. One way to generate single colonies is to titer a culture with serial dilutions and to pick colonies from one of the dilution plates. In this procedure, a small, measured amount of a bacterial culture is diluted into fresh liquid in another tube. A small amount of liquid is taken from this tube and diluted into another fresh tube. This process is repeated several times. Equal volumes of liquid are then taken from each of the dilution tubes and plated on petri plates. The plates are incubated overnight at 37°C; well-separated single colonies will arise on some of the dilution plates. The number of living bacteria in the culture is calculated from the number of colonies formed on the dilution plates.

A typical saturated culture contains 10^9 cells/ml. Phage suspensions can also be titered; a concentrated phage stock might typically contain 10^{11} phage/ml.

Materials

LB medium (*UNIT 1.1*)
LB plates (*UNIT 1.1*)
Sterile 16- or 18-mm-diameter culture tubes

1. Use pipets to introduce 5 ml LB medium into three sterile culture tubes. Line the tubes up, or label them so that they can be distinguished.
2. Using a pipettor, transfer 5 µl from the suspension of cells into the first tube of LB medium. Set the vortexer to a mild setting and agitate the tube for 5 sec.
3. Put a new tip on the pipettor and transfer 5 µl from the first tube of LB medium into the second tube, and vortex the second tube. Take 5 µl from the second tube and repeat step 3 until you have serial dilutions in all three tubes.

   The first dilution tube now contains a 10^3-fold dilution, generated by diluting the culture by a factor of one thousand (i.e., it contains 10^{-3} as many cells/ml as were present in the original culture). The second tube contains a 10^6-fold dilution, generated by diluting the original culture by a factor of one million (i.e., it contains 10^{-6} as many cells/ml as the original culture), etc.

   Many investigators prefer to perform serial dilutions with different volumes and different factors of dilution. These parameters can be modified in steps 1 to 3.

4. Spread 100 µl of liquid from the culture and from each dilution tube onto separate, labeled, dry LB plates (as described on p. 1.3.2). Incubate overnight at 37°C.

   During this incubation, each living bacterial cell will grow into a separate colony on the plate.

5. Count the colonies from these plates. Since only 100 µl was plated from the undiluted culture and from each dilution tube, each plate has 1/10 as many colonies on it as were present in each milliliter of liquid in the corresponding tube. Therefore, one can determine the number of cells that were present per milliliter of the culture by counting the number of colonies on a plate, and then multiplying that number by 10 times the factor of dilution.

   For example, if 22 colonies were observed on the plate corresponding to the 10^6-fold dilution, then the number of living cells in each milliliter of the original culture was $22 \times 10 \times 10^6$, or 2.2×10^8 cells/ml.

6. Any of the single colonies may be saved for further use. Store plates at 4°C wrapped in parafilm or in the plastic sleeve in which the plates were supplied.

Commentary

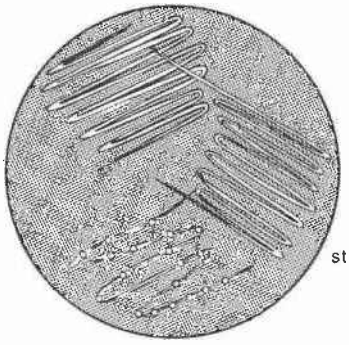
Titering by serial dilutions is a good way to determine the number of any kind of living organism present in a suspension. The organisms do not even need to be able to grow into colonies—i.e., the concentration of living bacteriophage in a tube can be determined by titering with serial dilutions and counting the number of plaques made when an aliquot of each dilution is plated on a lawn of phage-sensitive bacteria (see *UNIT 1.11*).

It is sometimes useful to use smaller factors of dilution. Mixing 50 µl of the culture into 5 ml of medium will give dilutions of 100×. Mixing 100 µl into 900 µl will give dilutions of 10×.

ISOLATING SINGLE COLONIES BY STREAKING A PLATE

BASIC PROTOCOL

Another way to isolate single colonies is called streaking or streaking for single colonies. This method is easier and faster than serial dilutions for isolating single colonies, but it cannot be used to count the number of cells in a culture. An inoculum of bacteria is streaked across one side of an agar plate with an inoculating loop or sterile toothpick. The resterilized loop or a fresh toothpick is then passed once through the first streak and streaked across a fresh part of the plate (see sketch 1.3A). This process is repeated at least once more, and the plate is incubated at 37°C until colonies become visible. If single colonies must be isolated from many bacteria, it is convenient to divide a plate into 4, 6, or 8 sectors and to streak for single colonies in each sector.

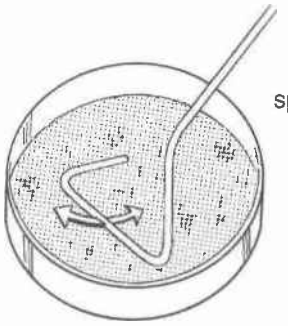


Sketch 1.3A

ISOLATING SINGLE COLONIES BY SPREADING A PLATE

BASIC PROTOCOL

It is sometimes necessary to distribute a liquid culture of bacteria evenly over the surface of a plate (for example, when plasmid-containing colonies are to be isolated after treatment of cells with plasmid DNA and calcium chloride, *UNIT 1.8*). This is usually done with a glass spreader. From 0.05 ml to 1 ml of liquid is pipetted onto a dry plate (see *UNIT 1.1*) and spread using a circular motion as shown in sketch 1.3B. Alternatively, the edge of the spreader can be used to make a raster pattern on the plate's surface. The plate can be turned at right angles and the process repeated. Evenly spread plates should be placed in the incubator with the lids ajar until they are completely dry.



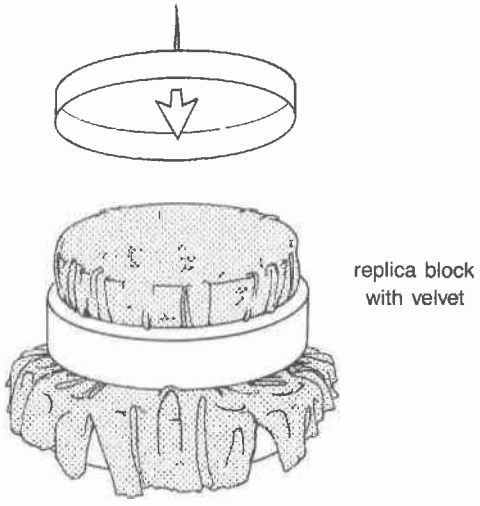
Sketch 1.3B

SUPPORT PROTOCOL

REPLICA PLATING

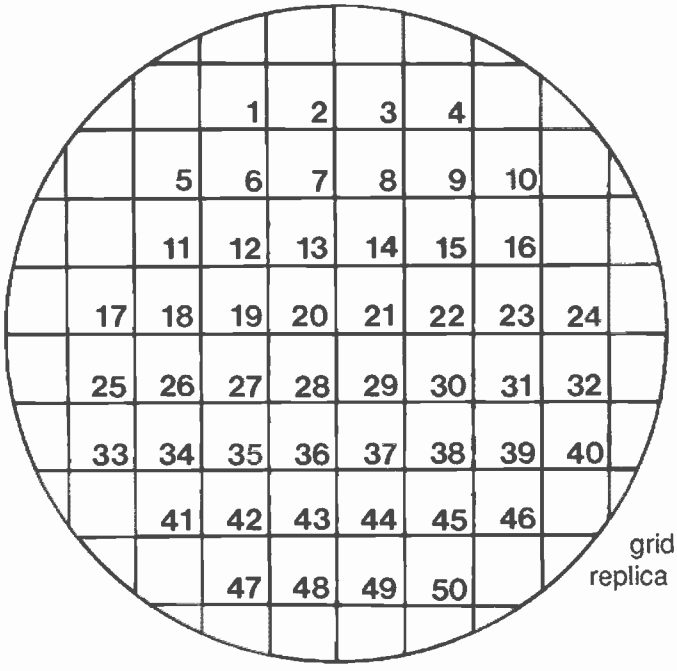
Replica plating is a convenient way to test many colonies for their ability to grow under different conditions. In this technique, bacterial colonies are transferred from one plate to another in a way that maintains the original pattern of colonies. This technique has many applications to recombinant DNA work. As an example, consider the plasmid pBR322, which contains two antibiotic resistance genes, encoding resistance to ampicillin and tetracycline (see Fig. 1.5.1). A piece of foreign DNA cloned into the tetracycline resistance gene inactivates it; cells that carry such a plasmid are ampicillin resistant but tetracycline sensitive. These cells can be identified by replica plating colonies from ampicillin-containing master plates onto plates containing tetracyline. Tetracyline-sensitive colonies can be identified by their inability to grow on the tetracycline plates, rescued from the master plate, and analyzed further.

This procedure requires two specialty items: a replica block and sterile velvets. The replica block is a wooden or metal cylinder that fits snugly inside a petri plate (see sketch 1.3C). One method for constructing these has been described by Adams (1965). A metal ring is used to secure the velvets to the block. Squares of velvet should be cut so as to cover the base (a diameter of 14 cm is suggested). These velvets can be washed, autoclaved, and reused. If velvets are not available, pieces of sterile filter paper or disposable replica plates can be used ("Repli-Plate" Colony Transfer Pads, American Laboratories #59901). Replica plating also requires a master plate composed of well-separated colonies. The master plate can be a fresh plate onto which 50 to 100 colonies have been gridded (using toothpicks and the grid in sketch 1.3D), or it can be a plate on which bacteria were spread that have now grown up into well-separated colonies.



Sketch 1.3C

Mark the top of the master plate to enable alignment with the grid. Press the plate down *lightly* onto the velvet. Do not bear down hard on the plate; pressing too hard will cause the colonies to run together on the velvet or may even cause the plate to collapse. Press new plates, oriented like the master plate, *lightly* onto the imprinted velvet to transfer the colonies. As many as 10 plates per velvet can often be replica plated.



Sketch 1.3D

STRAIN STORAGE AND REVIVAL

SUPPORT PROTOCOL

Most strains of *E. coli* can be stored for years in stab vials, or indefinitely if frozen at –70°C. It is prudent to check the genetic markers of a strain revived from storage. Ways to verify the presence of other selective markers are described in Table 1.4.4.

Stabs

Use airtight, autoclavable vials with rubber or Teflon caps (not cardboard). These are available from Wheaton Glassware and John's Scientific (¼-oz. Bijoux bottles, #15690-001). Fill the vials ⅔ full with stab agar (see recipe, *UNIT 1.1*). Inoculate them with a single colony (see sketch 1.3E) by collecting most of the cells in the colony with an inoculating loop, then repeatedly poking the loop deeply into the agar. Leave the cap of the stab vial slightly loose and incubate 8 to 12 hr at 37°C, or until cloudy tracks of bacterial growth are evident. Seal the vials tightly and store them in a cool (15° to 22°C), dark place. To revive a stored strain, flame sterilize an inoculating loop (*UNIT 1.1*), allow it to cool, insert it into the stab agar, and move the loop around until a gobbet of bacteria-laden agar is stuck onto the loop. Smear the gobbet onto one section of an LB plate and streak for single colonies (sketch 1.3A).

inoculating
a stab vial

Sketch 1.3E

Frozen Stocks

Add 2 ml of a mid-log culture or 1 ml of a freshly saturated culture to a stab vial or a Nunc vial (Nunc #1087) containing 1 ml glycerol solution or DMSO solution. Vials can be stored at –20° to –70°C, but most strains remain viable longer if stored at –70°C. Revive stored cells by scraping off splinters of solid ice with a toothpick or sterile pipet and streaking these splinters onto an LB plate. Do not allow the contents of the vial to thaw.

Glycerol solution

65% glycerol (vol/vol)
0.1 M $MgSO_4$
0.025 M Tris·Cl, pH 8

DMSO solution

7% dimethylsulfoxide (vol/vol)

The only advantage DMSO seems to have over glycerol for frozen stocks is that it is easier to pipet because it is less viscous. Use a bottle of reagent- or spectrophotometric-grade DMSO that has been kept tightly sealed.

LITERATURE CITED

Adams, J.N. 1965. Automotive pistons for use as bases in velveteen replication. *J. Bacteriol.* 89:1627.

Lederberg, J. and Tatum, E.L. 1953. Novel genotypes in mixed cultures of biochemical mutants of bacteria. *Cold Spring Harbor Symp. Quant. Biol.* 18:75.

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Massachusetts General Hospital and Harvard Medical School
Boston, Massachusetts

Selected Topics from Classical Bacterial Genetics

Current cloning technology exploits many facts learned from classical bacterial genetics. This unit covers those that are critical to understanding the techniques described in this book.

ANTIBIOTICS

Antibiotics are chemicals that kill microorganisms but are relatively nontoxic to eukaryotic organisms. Antibiotics are very important for the techniques described in this book; genes encoding resistance to them are carried on plasmid and phage vectors and cells that contain the vector are identified by their ability to grow and form colonies in the presence of the antibiotic. Table 1.4.1 gives stock and working concentrations, and mechanisms of action of most of the antibiotics that are used in recombinant DNA work.

Antibiotics are usually added to freshly autoclaved solid medium after it has cooled to below 50°C. In an emergency, antibiotics can be added directly to existing plates using the same final concentration as above (assume that a plate 100 mm in diameter contains a total medium volume of 30 ml). Allow the antibiotic time to diffuse away from the very surface of the plate; an hour is usually sufficient. Since many antibiotics (especially ampicillin) lose potency at room temperature, plates are usually stored at 4°C. In addition, rifampicin and tetracycline should be stored in the dark (see Table 1.4.1).

THE *LAC* OPERON

Many of the techniques described in this book were made possible by early studies of the *E. coli lac* operon. The *lac* operon consists of three genes—*lacZ*, *lacY*, and *lacA* (see Figure 1.4.1). When the cell grows on rich medium or glucose minimal medium,

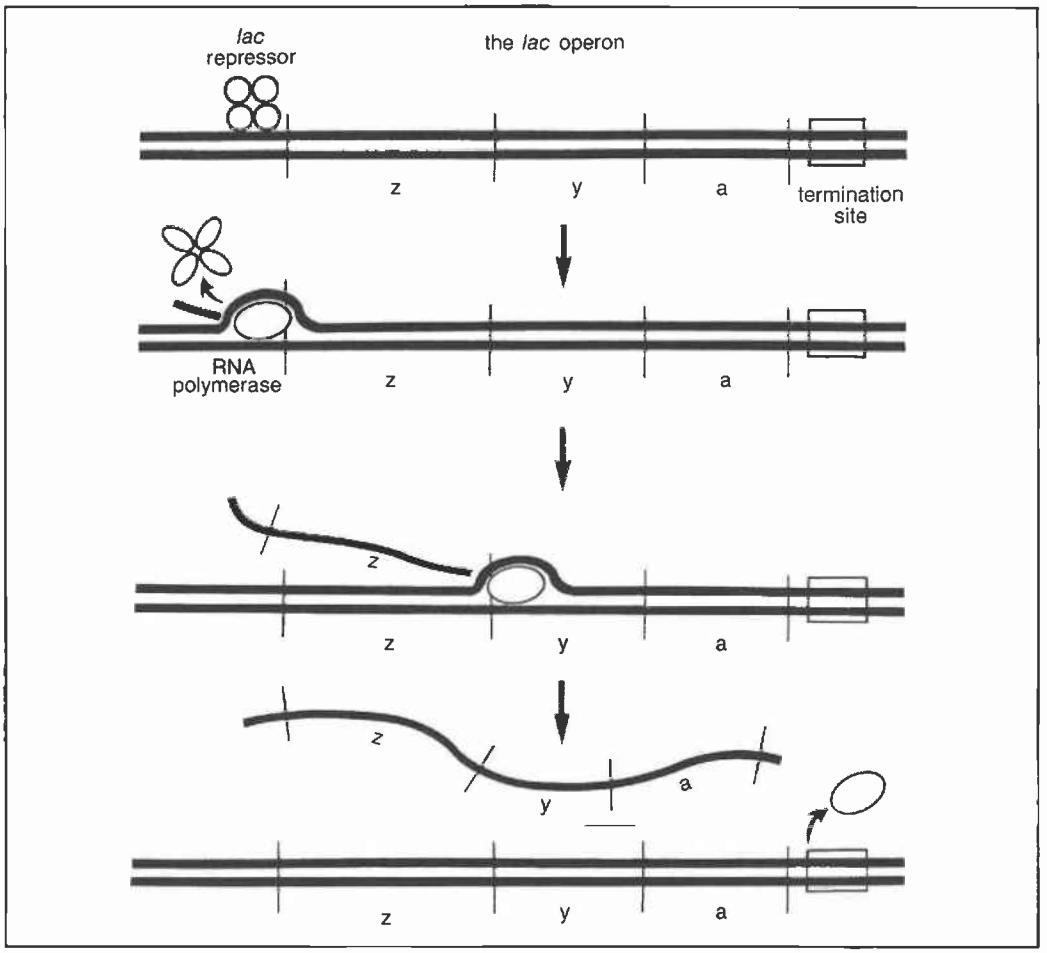


Figure 1.4.1 The first line shows the genes of the *lac* operon. *lacZ*, *lacY*, and *lacA* transcription is repressed by *lac* repressor. In the next line, inactive repressor is no longer bound to the operator, and the genes are being transcribed by RNA polymerase. In the third line, RNA polymerase transcribes the operon. Finally, RNA polymerase encounters a transcription terminator and, in a reaction requiring rho protein, RNA polymerase is detached from the DNA and the nascent transcript.

Table 1.4.1 Antibiotics, Their Modes of Action, and Modes of Bacterial Resistance[a]

Antibiotic[b]	Stock conc. (mg/ml)	Final conc. (μg/ml)	Mode of action	Mode of resistance
Ampicillin[c]	4	50	Bacteriocidal; only kills growing *E. coli*; inhibits cell wall synthesis by inhibiting formation of the peptidoglycan cross-link	β-lactamase hydroylzes ampicillin before it enters the cell
Chloramphenicol, in methanol	10	20	Bacteriostatic; inhibits protein synthesis by interacting with the 50S ribosomal subunit and inhibiting the peptidyltransferase reaction	Chloramphenicol acetyltransferase inactivates chloramphenicol
D-Cycloserine,[d] in 0.1 M sodium phosphate buffer, pH 8	10	200	Bacteriocidal; only kills growing *E. coli;* inhibits cell wall synthesis by preventing formation of D-alanine from L-alanine and formation of peptide bonds involving D-alanine	Mutations destroy the D-alanine transport system
Gentamycin	10	15	Bacteriocidal; inhibits protein synthesis by binding to the L6 protein of the 50S ribosomal subunit	Aminoglycoside acetyltransferase and aminoglycosidenucleotidyltransferase inactivate gentamycin; mutations in *rplF* (encodes the L6 protein) prevent the gentamycin from binding
Kanamycin	10	30	Bacteriocidal; inhibits protein synthesis; inhibits translocation and elicits miscoding	Aminoglycoside phosphotransferase, also known as neomycin phosphotransferase, aminoglycoside acetyltransferase, and aminoglycoside nucleotidyltransferase; inactivates kanamycin
Kasugamycin	10	1000	Bacteriocidal; inhibits protein synthesis by altering the methylation of the 16S RNA and thus an altered 30S ribosomal subunit	Mutations prevent kasugamycin from binding to the ribosome; mutations decrease uptake of kasugamycin
Nalidixic acid, pH to 11 with NaOH	5	15	Bacteriostatic; inhibits DNA synthesis by inhibiting DNA gyrase	Mutations in the host DNA gyrase prevent nalidixic acid from binding
Rifampicin,[e] in methanol	34	150	Bacteriostatic; inhibits RNA synthesis by binding to and inhibiting the β subunit of RNA polymerase; rifampicin sensitivity is dominant.	Mutation in the β subunit of RNA polymerase prevents rifampicin from complexing; rifampicin resistance is recessive
Spectinomycin	10	100	Bacteriostatic; inhibits translocation of peptidyl tRNA from the A site to the P site	Mutations in *rpsE* (encodes the S5 protein) prevent spectinomycin from binding; spectinomycin sensitivity is dominant and resistance is recessive
Streptomycin	50	30	Bacteriocidal; inhibits protein synthesis by binding to the S12 protein of the 30S ribosomal subunit and inhibiting proper translation; streptomycin sensitivity is dominant	Aminoglycoside phosphotransferase inactivates streptomycin; mutations in *rpsL* (encodes the S12 protein) prevent streptomycin from binding; streptomycin resistance is recessive
Tetracycline,[e] in 70% ethanol	12	12	Bacteriostatic; inhibits protein synthesis by preventing binding of aminoacyl tRNA to the ribosome A site	Active efflux of drug from cell

[a]Data assembled from Foster (1983), Gottlieb and Shaw (1967), and Moazed and Noller (1987).

[b]All antibiotics should be stored at 4°C, except tetracycline, which should be stored at –20°C. All antibiotics should be dissolved in sterile distilled H_2O unless otherwise indicated.

[c]Carbenicillin, at the same concentration, can be used in place of ampicillin. Carbenicillin can be stored in 50% ethanol/50% water at –20°C.

[d]D-cycloserine solutions are unstable. They should be made immediately before use.

[e]Light-sensitive; store stock solutions and plates in the dark.

Table 1.4.2 Lactose Analogs Used in DNA Cloning Technology

Galactoside	Stock concentration[a]	Use	Characteristics	Reference
Isopropyl-1-thio-β-D-galactoside (IPTG)	100 mM	Very effective inducer	Nonmetabolizable inducer	Barkley and Bourgeois, 1978 (pp. 177-220)
5-Bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal)	20 mg/ml (dissolved in *N,N* dimethyl formamide)	Identification of *lacZ*$^+$ bacteria, especially useful for detecting β-galactosidase made by recombinant vectors	Noninducing chromogenic substrate of β-galactosidase (cleavage of Xgal results in blue color); production of bluecolor independent of *lacY* gene product	Miller, 1972
Orthonitrophenyl-β-D-galactoside (ONPG)	10 mM	β-galactosidase assays	Chromogenic substrate of β-galactosidase (cleavage of ONPG results in yellow color)	Miller, 1972 (pp. 352-355)
6–O–β-D-Galacto-pyranosyl D-glucose (allolactose)			Inducer of the lactose operon in vivo; lactose is converted into allolactose by β-galactosidase	Zabin and Fowler, 1978 (pp. 89-121)
Phenyl-β-D-galacto-side (Pgal)	2 mg/ml	Selection for *lac* constitutive mutants	Noninducing substrate of β-galactosidase; uptake partly dependent on *lacY* gene product	Miller, 1978 (pp. 31-88)
Orthonitrophenyl-β-D-thiogalacto-side (TONPG)	10 mM	Selection for *lac*$^-$ mutants	Transported into cells by *lac* permease (the *lacY* gene product); inhibits cell growth at high concentration	Miller, 1978 (pp. 31-88)

[a]Stock solutions should be dissolved in sterile water unless otherwise noted.

transcription is blocked by *lac* repressor (product of the neighboring *lacI* gene) which binds to a single site (operator) upstream of *lacZ* and prevents RNA polymerase from binding to the promoter. When the cell grows on medium that contains lactose or certain related compounds (see Table 1.4.2), *lac* repressor no longer binds the operator, and RNA polymerase synthesizes a single mRNA which encodes *lacZ*, *lacY*, and *lacA*. (In the wild-type *lac* operon, transcription initiation also requires the presence of a cAMP-CRP activator complex; all *lac* promoters used in cloning experiments are independent of this control.) Two of these genes are necessary for growth on lactose. *lacY* encodes a permease which is necessary for the uptake of lactose and certain related sugars. *lacZ* encodes a β-galactosidase, which cleaves lactose into glucose and galactose, which the cell then utilizes. The third structural gene, *lacA*, encodes an enzyme called thiogalactoside transacetylase. This enzyme is not required for lactose metabolism. Its function during growth on lactose-containing medium is not clear.

Alpha-Complementation

Vectors such as the pUC series and the M13mp series (see *UNITS 1.5, 1.14, & 1.15*) contain a piece of DNA that encodes an alpha fragment of β-galactosidase. These vectors exploit a phenomenon called *alpha-complementation* (see sketch 1.4A), which was discovered by Ullman, Jacob, and Monod in 1967. They showed that a cell that bears any of a number of deletions of the 5′ end of the *lacZ* gene synthesizes an inactive C-terminal fragment of β-galactosidase, called an *omega (ω) fragment*. Similarly, a cell that bears a deletion of the 3′ end of *lacZ* encodes an inactive N-terminal fragment of β-galactosidase called an *alpha (α)* fragment. However, if a cell contains two genes, one directing the synthesis of an alpha fragment, the other directing synthesis of an omega fragment, the β-galactosidase activity is observed. Many vectors incorporate a *lac* α-fragment gene, which is small and easily manipulated. Exploitation of these vectors requires use of a strain carrying the complementing ω-fragment gene to allow assembly of an active complex. This

gene is often carried on an F′ plasmid (see below). When these vectors are used, cells containing them are grown on medium containing IPTG, which inactivates *lac* repressor and thus derepresses ω peptide synthesis, and Xgal, which is turned blue by the enzymatic activity of β-galactosidase (see Table 1.4.2). On this medium, these vector-containing cells possess β-galactosidase activity and turn blue.

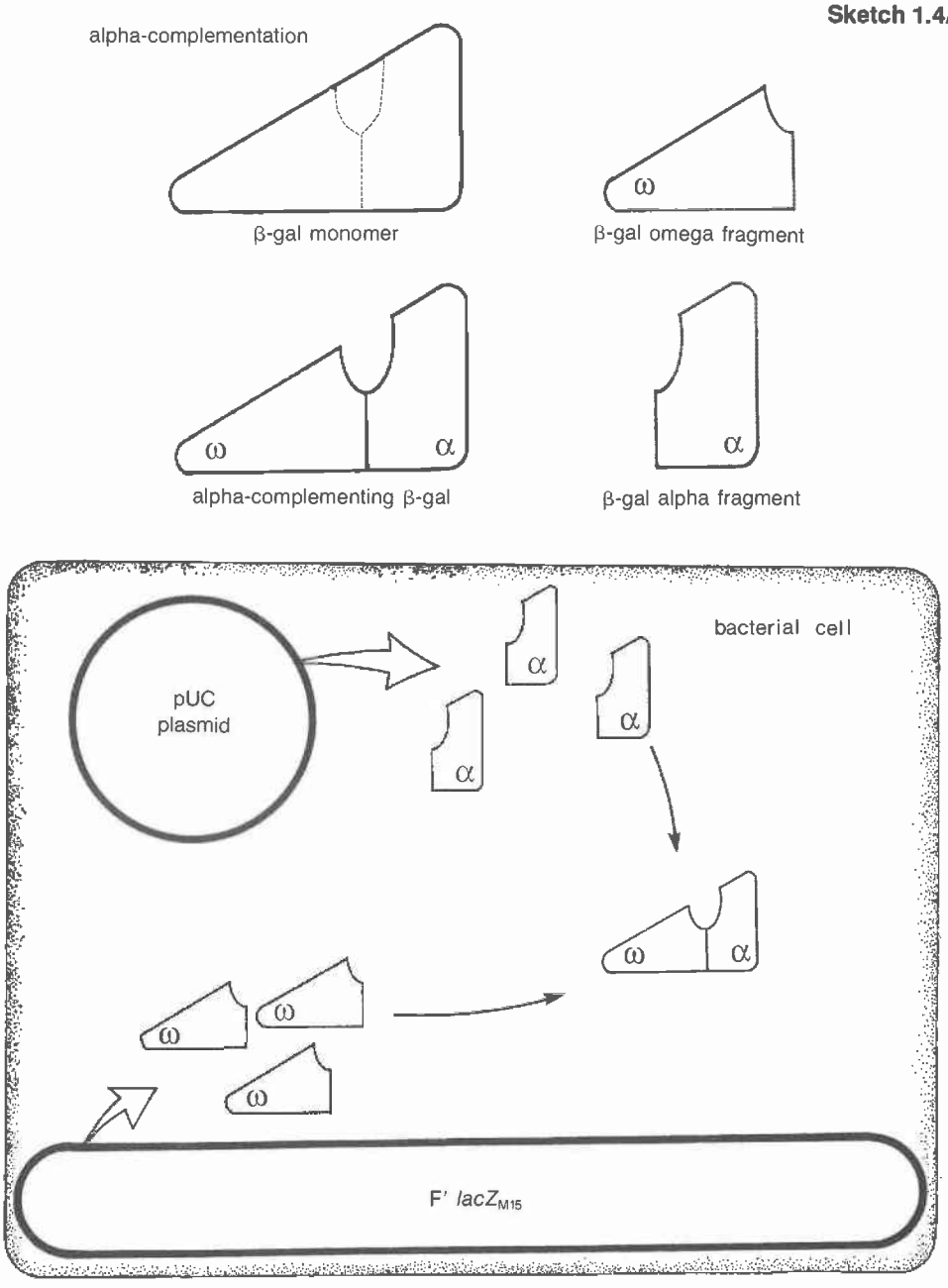
Lactose Analogs

There are many compounds related to lactose that were first used for the biochemical and genetic analysis of *lac* operon activity and are used in the cloning technology described in this book. These are described in Table 1.4.2.

THE F FACTOR

The F (fertility) factor is a genetic unit found in some strains of *E. coli*. Bacteria that contain the F factor are used in many techniques described in this book, mainly for two reasons. First (as described later in this chapter), possession of F allows a cell to be infected by vectors based on filamentous phages, which bind to cell surface structures called pili elaborated by F-containing cells. Second, defective *lacZ* genes that encode the ω fragment of β-galactosidase (described above)

Sketch 1.4A



are commonly carried on F′ factors.

The F factor is found in three alternative forms: as double-stranded, single-copy, circular extrachromosomal plasmid DNA (F^+); as plasmid DNA like F^+ but also including other bacterial genes (F′); and as a stretch of linear DNA integrated into various sites on the bacterial chromosome (Hfr). Possession of the F factor confers on *E. coli* the ability to donate DNA in bacterial crosses (or matings). For this reason cells that carry F are sometimes called *male*. F or F′ plasmids can transfer themselves to other cells, and may occasionally cause transfer of other plasmids. This latter process is called *mobilization*. Mutations called *tra* prevent F from transferring itself or mobilizing other plasmids. Integrated Tra^+ F factors (Hfr) can cause transfer of chromosomal DNA to other cells, but the recipient usually does not receive the F sequence.

NONSENSE SUPPRESSORS

Some vectors used in recombinant DNA research (e.g., plasmid πVX and phage Charon 4a) contain nonsense mutations in essential genes. These vectors must be propagated in special strains of *E. coli*. In these strains, translation of messages does not always stop when the ribosome encounters a chain termination codon (amber or ochre), but sometimes continues, with a new amino acid installed at the end of the growing polypeptide. This process is called *nonsense suppression* and strains of *E. coli* in which it occurs are said to contain *nonsense suppressors*. In a strain that contains an efficient or a "strong" suppressor, suppression might occur 50% of the time an amber codon is encountered.

The mechanism of nonsense suppression is a simple one: the cell contains a mutant species of tRNA in which the anticodon loop has mutated so that it base pairs with the UAG amber codon or the UAA ochre codon. Nonsense suppressors commonly used in cloning technology are given in Table 1.4.3. UGA (opal) suppressors also exist but are rarely used.

GENETIC MARKERS

Genetic markers in *E. coli* are named according to the convention proposed by Demerec et al. (1966). All genes of a given strain are presumed to be in the wild-type state unless otherwise noted in the genotype (see box). Gene names have three italicized lowercase letters, sometimes followed by an italic uppercase letter, and sometimes also followed by an italic arabic number that specifies the precise mutation (allele) in question (e.g., *lacY1*, *trp-31*). The three-letter combination is usually a mnemonic intended to suggest the function of the gene. Proper notation omits superscript + and – in a genotype, but these are sometimes used redundantly for clarity. Deletion mutations are described by Δ, followed by the names of deleted genes in parentheses, followed by the allele number [e.g., Δ(*lac-pro*)X111]. The delta may be replaced by "del" or "d." Sometimes a phenotype designation (see box) in parentheses follows the genotype designation, if the former is not obvious from the latter [e.g., *rpsL104* (Str^r)]. However, this usage is by no means universal.

Table 1.4.4 lists commonly used genetic markers, with methods for verifying their presence or absence in bacterial cells. Genotypes of several strains used for different applications are listed in Table 1.4.5.

Table 1.4.3 Commonly Used Nonsense Suppressors[a]

Suppressor	Map position[b]	Type of suppressor	Amino acid inserted	tRNA gene
supD (sul)	43	Amber	Serine	*serU*
supE (su2)	16	Amber	Glutamine	*glnU*
supF (su3)	27	Amber	Tyrosine	*tyrT*
supB (suB)	16	Ochre/amber	Glutamine	*glnU*
supC (suC)	27	Ochre/amber	Tyrosine	*tyrT*

[a]Data compiled from Bachmann (1983) and Celis and Smith (1979).
[b]Given in minutes; see Bachmann (1983) for description.

GENOTYPE AND PHENOTYPE

Genotype indicates what genes are mutated in a strain. A genotype is a theoretical construct describing a genetic constitution that would explain the phenotype of the strain. It is derived from considerations of the strain's behavior and ancestry.

Phenotype describes the observable behavior of the strain—e.g., Lac$^-$ fails to grow on lactose as a sole carbon source. Phenotypes are in Roman type, the first letter is capitalized, and the letters are always followed by superscript + or – (sometimes r, resistant, or s, sensitive). A phenotype is a datum to be explained.

Genotype and phenotype names are usually related, but the relationship is not always obvious. Examples are provided below.

Genotype	Phenotype	Description of phenotype
Some straightforward examples:		
trp-31	Trp$^-$	Requires tryptophan for growth on minimal media
uvrA	UV^s	Sensitive to UV light
recA	Rec$^-$	Recombination defective
Some common examples that are not so straightforward:		
supE44	Sup$^+$	Carries a tRNA suppressor gene. The *mutant* gene product, not the wild type, suppresses nonsense mutations; wild type is indicated as *sup^0*, Sup$^-$, and does not suppress
rpsL104	Str^r	Resistant to streptomycin (this makes a mutant *r*ibosomal *p*rotein, *s*mall subunit, the target of the drug)
rpsE	Spc^r	Resistant to spectinomycin (also codes for a *r*ibosomal protein, a different one)
gyrA	Nal^r	Resistant to nalidixit acid (the mutation affects DNA gyrase)
One mutation may create several phenotypes:		
dam-3	Dam$^-$, 2-AP^s UV^s	DNA not methylated at adenines in GATC Sensitive to 2-aminopurine Sensitive to UV light
hsdS	*Eco*K R$^-$, *Eco*K M$^-$	Neither restricts nor modifies DNA that enters the cell
Some mutations lead to counterintuitive phenotypes:		
recD	ExoV$^-$ but Rec$^+$	Exonucleolytic activity of the RecBCD protein is defective, but the recombinational activity is intact

DNA RESTRICTION, MODIFICATION, AND METHYLATION

This section and the next two describe *E. coli* functions that can prevent cloning the sequence of interest. *E. coli* has at least four restriction systems that identify foreign DNA and destroy it. These systems, encoded by *hsdRMS*, *mcrA*, *mcrB*, and *mrr*, can be avoided by using host strains in which they are disabled by mutation. Restriction of DNA and the content of methylated bases in the DNA are interrelated as described below. To select the appropriate strain, it is necessary to know the content of methylated bases in the DNA to be cloned.

The *Eco*K restriction system, encoded by the *hsdRMS* genes, is the best understood of

Table 1.4.4 Commonly Used Genetic Markers and How to Test Them[a]

Nutritional markers	Streak or replica plate colonies of the strain onto plates with and without the nutrient to be tested, but which contain all other necessary nutrients.
Antibiotic resistance markers	Streak or replica plate colonies of the strain onto plates with and without the antibiotic.
Other markers	
lacZ$^+$	Streak strain on an LB plate with Xgal and IPTG (*UNIT 1.4*). Colonies should turn blue. Colonies of control *lacZ*$^-$ strain should not turn blue.
lacZΔM15[b]	Transform strain with pUC plasmid and with control plasmid such as pBR322. Streak transformants onto LB/ampicillin plate with Xgal and IPTG. Colonies bearing pUC plasmid should turn blue, while colonies bearing pBR322 should not.
F$^+$ or F′	Spot M13 phage onto a lawn of the cells. Small plaques should appear (see *UNIT 1.15*).
recA	Using a toothpick, make a horizontal stripe of cells across an LB plate. Also make a stripe of *recA*$^+$ control cells. Cover half of the plate with a piece of cardboard, and irradiate the plate with 300 ergs/cm^2 of 254 nm UV light from a hand-held UV source (typically 20 sec exposure from a lamp held 50 cm over the plate). *recA*$^-$ cells are very sensitive to killing by UV light, and *recA*$^-$ cells in the unshielded part of the plate should be killed by this level of irradiation.
recBCD	Spot dilutions of λ gam$^-$(*UNIT 1.9*) on a lawn of cells side by side with dilutions of λ gam$^+$. The *gam*$^-$ plaques should be almost as big as the *gam*$^+$ plaques.
hsdS$^-$	(1) Use the strain and a wild-type strain to plate out serial dilutions of a λ-like phage stock grown on an *hsdS*$^-$ or *hsdR*$^-$ host. If the phage stock came from an *hsdS*$^-$ host, then it should make plaques with 10^4 to 10^6 higher efficiency on the putative *hsdS*$^-$ host than on a wild-type host. If the plate stock came from an *hsdR*$^-$ (*hsdS*$^+$ *hsdM*$^+$) host, it should make plaques with the same efficiency on both strains. (2) Suspend one of the fresh plaques from the putative *hsdS*$^-$ host in 1 ml lambda dilution buffer. Titer this suspension on the putative *hsdS*$^-$ strain and on a wild-type strain. The suspension should make plaques at 10^4 to 10^6 higher efficiency on the *hsdS*$^-$ strain than on the wild-type strain. One plaque contains ~10^7 phage.
hsdR$^-$ (*hsdS*$^+$ *hsdM*$^+$)	(1) Perform step 1 described above, using a plate stock made from an *hsdS*$^-$ host. (2) Suspend one of the fresh plaques in 1 ml lambda dilution buffer. Titer this suspension on the putative *hsdR*$^-$ strain and on a wild-type strain. This suspension should make plaques with the same efficiency on the *hsdR*$^-$ as on a wild-type strain.
dam	Transform the strain and a wild-type strain with a plasmid that contains recognition sites for the enzymes *Mbo*I or *Bcl*I. Prepare plasmid DNA from both strains and verify that plasmid DNA isolated from the *dam*$^-$ strain is sensitive to digestion by the enzyme.
dcm	Transform the strain and a wild-type strain with a plasmid that contains recognition sites for *Scr*FI. Prepare plasmid DNA from both strains to verify that only plasmid DNA from the *dcm* strain is fully sensitive to digestion by the enzyme. Half of the *Scr*FI sites will be cut even when the DNA is *dcm*-methylated.
lon	Streak LB plate for single colonies. Also streak a control plate of a wild-type strain. Incubate at 37°C. Colonies of the *lon*$^-$ strain should be larger, glistening, and mucoidal.

[a]Commonly used protocols in this table are media preparation (*UNIT 1.1*), streaking and replicating a plate (*UNIT 1.3*), and growing lambda-derived vectors (*UNIT 1.12*).

[b]Encodes omega fragment of β-galactosidase.

Table 1.4.5 Commonly Used *Escherichia coli* Strains

Strain[a]	Genotype	Reference[b]
AR58	*sup*^{0} *galK2 galE*::Tn*10* (λcI857 ΔH1 *bio*$^-$ *uvrB kil*$^-$ *cIII*$^-$) Str^r	A. Shatzman, pers comm.†
AR120	*sup*^{0} *galK2 nad*::Tn*10* (Tet^r) (λcI$^+$ *ind*$^+$ p_L-*lacZ fusion*) Str^r	A. Shatzman, pers comm.†
AS1[c]	*endA1 thi-1 hsdR17*($r_K^-m_K^+$) *supE44* (λcI$^+$)	A. Shatzman, pers comm.†
BNN102[c]	C600 *hflA150 chr*::Tn*10 mcrA1 mcrB*	Young and Davis, 1983*
BW313[d]	*Hfr lysA*$^-$ *dut ung thi-1 recA spoT1*	Kunkel et al., 1987*†
C600	*thi-1 thr-1 leuB6 lacY1 tonA21 supE44 mcrA*	Appleyard, 1954*‡
CJ236[d]	*dut1 ung1 thi-1 relA1*/pCJ105 (Cm^r)	Kunkel et al., 1987*; Joyce and Grindley, 1984+
DH1	*recA1 endA1 thi-1 hsdR17 supE44 gyrA96* (Nal^r) *relA1*	Hanahan, 1983*; D. Hanahan, pers. comm.†‡
DH5αF′[e]	F′/*endA1 hsdR17*($r_K^-m_K^+$) sup*E44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)$_{U169}$ (*m80lacZΔM15*)	See DH1 references
DK1	*hsdR2 hsdM*$^+$ *hsdS*$^+$ *araD139* Δ(*ara-leu*)$_{7697}$ Δ(*lac*)$_{X74}$ *galU galK* rpsL (Str^r) *mcrA mcrB1* Δ(*sr1-recA*)$_{306}$	D. Kurnit and B. Seed, pers. comm.†‡
ER1451	F′ *traD36 proAB lacI*^{q} Δ(*lacZ*)*M15*/*endA gyrA96 thi-1 hsdR2* (or *hsdR17*) *supE44* Δ(*lac-proAB*) *mcrB1 mcrA*	Raleigh et al., 1988†‡
HB101[f]	Δ(*gpt-proA*)*62 leuB6 thi-1 lacY1 hsdS*$_B$*20 recA rpsL20* (Str^r) *ara-14 galK2 xyl-5 mtl-1 supE44 mcrB*$_B$	Boyer and Roulland-Dussoix, 1969*†‡
JM101[g]	F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*Δ*M15*/*supE thi* Δ(*lac-proAB*)	Yanisch-Perron et al., 1985*†‡
JM105[g]	F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*Δ*M15*/Δ(*lac-pro*)$_{XIII}$ *thi rpsL* (Str^r) *endA sbcB supE hsdR*	See JM101 references
JM107[g]	F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*Δ*M15*/*endA1 gyrA96* (Nal^r) *thi hsdR17 supE44 relA1* Δ(*lac-proAB*) *mcrA*	See JM101 references
JM109[g]	F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*Δ*M15*/*recA1 endA1 gyrA96* (Nal^r) *thi hsdR17 supE44 relA1* Δ(*lac-proAB*) *mcrA*	See JM101 references
K38	HfrC (λ)	Russel and Model, 1984; see *UNIT 16.2*
KM392	*hsdR514*($r_K^-m_K^+$) *supE44 supF58 lacY galK2 galT22 metB1 trp55 mcrA* Δ*lac*$_{U169}$ *proC*::Tn*5*	T. St. John, pers. comm.†; K. Moore‡
LE392	*hsdR514*($r_K^-m_K^+$) *supE44 supF58 lacY galK2 galT22 metB1 trp55 mcrA*	Borck, et al., 1976*; N. Murray, pers. comm.†; L. Enquist‡

continued

the *E. coli* restriction mechanisms (Bickle, 1982). It attacks DNA that carries the site:

5′ A ^{m}A C N N N N N G T G C 3′
3′ T T G N N N N N C ^{m}A C G 5′

and results in double-strand cleavage at a variable distance from the site, leading eventually to degradation of the resulting fragments. DNA is not attacked if it lacks the site, or if the site is present but methylated at the adenines shown (^{m}A).

The *Eco*K enzyme is both genetically and enzymatically complex. The HsdR, HsdM, and HsdS subunits are required for restriction of an unmethylated substrate. The same complex will methylate the same substrate, but at a very slow rate, so that an unmethylated target rarely survives. A substrate methylated on only one strand (hemimethylated) will be methylated on the other strand by the three-protein complex, but will not be cut. HsdM and HsdS together can methylate either an unmethylated or a hemimethylated substrate. The three-protein complex is inactive for restriction if any of the three subunits is defective, but can still methylate if HsdR is defective.

In summary, a strain defective in the *hsdR* gene is described as having the phenotype HsdR$^-$M$^+$ (or, equivalently, *Eco*K R$^-$M$^+$ or $R_{\bar{K}}M_{\bar{K}}^{\ddagger}$; see box): it will methylate newly introduced DNA but will not restrict it. However, a strain defective in either *hsdM* or *hsdS* will neither restrict nor methylate, and has the phenotype HsdR$^-$M$^-$ (or *Eco*K R$^-$M$^-$ or $R_{\bar{K}}M_{\bar{K}}$.

In contrast with *Eco*K, the other three restriction systems of *E. coli* K-12—*mcrA*, *mcrB*,

Table 1.4.5 Commonly Used *Escherichia coli* Strains, continued

Strain[a]	Genotype	Reference[b]
MC1061	*hsdR2 hsdM⁺ hsdS⁺ araD139* $\Delta(ara\text{-}leu)_{7697}$ $\Delta(lac)_{X74}$ *galE15 galK16 rpsL* (Str^r) *mcrA mcrB1*	Casadaban and Cohen, 1980*; M. Casadaban†‡
MM294	*endA thiA hsdR17 supE44*	Backman et al., 1976*; M. Meselson†‡
NM539[h]	*supF hsdR* (P2*cox*3)	Frischauf et al., 1983*; Lindahl and Sunshine, 1972†; N. Murray‡
P2392	*hsdR514*($r_K^-m_K^+$) *supE44 supF58 lacY galK2 galT22 metB1 trp55 mcrA* (P2)	L. Klickstein, pers. comm.†
PR722	F′ $\Delta(lacIZ)_{E65}$ *pro⁺/proC*::Tn*5* $\Delta(lacIZYA)_{U169}$ *hsdS20 ara-14 galK2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44 leu*	P. Riggs, pers.comm.†
Q359	*hsdR⁻ hsdM⁺ supE tonA* (φ80^r) (P2)	Karn et al., 1980*†‡
RR1	Δ*(gpt-proA)62 leuB6 thi-1 lacY1* $hsdS_B$*20 rpsL20* (Str^r) *ara-14 galK2 xyl-5 mtl-1 supE44* $mcrB_B$	Bolivar et al., 1977; see *UNIT 16.5*
Y1088[i]	*supE supF metB trpR hsdR⁻ hsdM⁺ tonA21 strA* Δlac_{U169} *mcrA proC*::Tn*5*/pMC9	Huynh et al., 1985*; Miller et al., 1984†; R. Young‡; M. Calos (pMC9)‡
Y1089[i]	Δlac_{U169} *proA⁺* Δ*(lon) araD139 strA hflA150 chr*::Tn*10*/pMC9	See Y1088 references
Y1090[i]	Δlac_{U169} *proA⁺* Δ*(lon) araD139 strA supF trpC22*::Tn*10 mcrA*/pMC9	See Y1088 references

[a]The original *E. coli* K-12 strain was an F⁺ λ lysogen, but most K-12 derivatives in common use have been cured of the F factor and prophage and these are indicated only when present. All other genes in these strains are presumed to be wild-type except for the genotype markers noted in the second column.

[b]Reference for all *mcr* and *mrr* genotypes is Raleigh et al., 1988. Specific information for each strain can be found as indicated by: * reference for genotype of strain; † source of additional genotype information; ‡ thought to be responsible for original strain construction.

[c]AS1 is also known as MM294cI⁺. BNN102 is also known as C600 *hflA*.

[d]Both CJ236 and BW313 are commonly used in oligonucleotide-directed mutagenesis. pCJ105, the plasmid CJ236 carries, is not relevant for this application.

[e]Three strains are in circulation. DH5 is a derivative of DH1 that transforms at slightly higher efficiency. DH5α and DH5αF′ are derivatives that carry a deletion of the lac operon and a Φ80 prophage that directs synthesis of the omega fragment of β-galactosidase. DH5αF′ carries an F′ factor as well. DH5α and DH5αF′ are proprietary strains and the cells are prepared in some way that allows them to be transformed with slightly higher efficiency than DH5.

[f]In this strain, the area of the chromosome that contains the *hsd* genes was derived from the related B strain of *E. coli*.

[g]The continued presence of the F′ factor in JM strains can be insured by starting cultures only from single colonies grown on minimal plates that do not contain proline. These strains encode the omega fragment of *lacZ* and are frequently used with vectors that direct the synthesis of the *lacZ* alpha fragment. These strains are frequently used with M13 vectors for DNA sequencing (*UNITS 1.14, 1.15, & 7.4*).

[h]It is not known whether this strain has markers other than those listed.

[i]pMC9, the plasmid in the Y strains listed here, directs the synthesis of large amounts of *lac* repressor. It also confers resistance to tetracycline and ampicillin (Lebrowski et al., 1984, *EMBO. J.* 3:3117-3121).

and *mrr*—specifically attack DNA that *is* methylated at particular sequences, rather than DNA that is not. Either methylated cytosine residues or methylated adenine residues can create problems (see below).

The action of either *mcrA* or *mcrB* reduces the number of clones recovered from libraries made with genomic DNA from other organisms, and leads to bias against recovery of specific fragments from those libraries (Raleigh et al., 1988; Whittaker et al., 1988; Woodcock et al., 1988, 1989; *mrr* has not been tested). For McrB there is evidence that a nuclease is responsible for these effects (E. Sutherland and E.A. Raleigh, unpublished observation), but no such evidence is available for the other two systems.

Even without biochemical characterization, something can be said of the recognition sites for these systems. McrA restricts DNA modified by the *Hpa*II (5′ C^{m}CGG) methylase and possibly other methylases. McrB restricts DNA modified by any one of 14 other modification methylases, which led to the suggestion that the McrB recognition site is 5′ G^{m}C (Raleigh and Wilson, 1986). Mrr restricts DNA modified by the *Hha*II (5′ G^{m}ANTC) or *Pst*I (5′ CTGC^{m}AG) methylases, but *not* that modified by the *Eco*RI methylase, among others (Heitman and Model, 1987).

Many commonly used *E. coli* strains are McrA⁻; including (from Table 1.4.5) BNN102 (also known as C600*hflA*), C600, JM107, JM109, LE392, Y1088, and Y1090. Of the strains listed in Table 1.4.5, only BNN102,

HB101, and MC1061 are McrB$^-$; and only HB101 is Mrr$^-$ (see also Raleigh et al., 1988).

A strain should be used which lacks the appropriate methyl-specific restriction system(s) when cloning genomic DNA from an organism containing methylated bases. All mammals and higher plants, and many prokaryotes, contain methylcytosine (Ehrlich and Wang, 1981), so McrA$^-$B$^-$ strains should be used for libraries of DNA from these organisms. Bacteria and lower eukaryotes may contain methyladenine, so Mrr sensitivity should be considered. However, the important experimental organisms *Drosophila melanogaster* and *Saccharomyces cerevisiae* contain no detectable methylated bases.

In addition, any time DNA is methylated in vitro during a manipulation, an appropriate restriction-deficient host should be used as DNA recipient. Methylases are used to generate novel restriction enzyme specificities or to protect cDNA from subsequent digestion (see *UNITS 3.1 & 5.6*). For example, the *Alu*I methylase (M.*Alu*I) is sometimes used to protect *Hin*dIII sites. McrB will restrict DNA modified by M.*Alu*I.

Once the DNA introduced into *E. coli* has been replicated, the foreign methylation pattern will be lost (and the *E. coli* methylation pattern will be acquired) unless the clone carries a methylase activity. Once successfully introduced, clones can be freely transferred among Mcr$^+$ Mrr$^+$ *E. coli* strains, since the methylation pattern will no longer be foreign. It is important that the clone be passed through an HsdM$^+$ strain before trying to introduce it into an HsdR$^+$ strain.

The normal methylation pattern of *E. coli* DNA is the product of three methylases. The *Eco*K methylase modifies the sequences indicated above. The *dam* and *dcm* gene products are also methylases (Marinus, 1987). The recognition sites for these are:

dam	5′	G	^{m}A	T	C	3′
	3′	C	T	^{m}A	G	5′
dcm	5′	C	^{m}C	A	G	G	3′
	3′	G	G	T	^{m}C	C	5′
dcm	5′	C	^{m}C	T	G	G	3′
	3′	G	G	A	^{m}C	C	5′

These modifications will render DNA resistant or partially resistant to some restriction endonucleases used for in vitro work (see Table 3.1.1), such as *Mbo*I and *Bcl*I (for Dam-modified DNA) or *Eco*RII (for Dcm-modified DNA). The Dam and Dcm methylases are *not* associated with any *E. coli* restriction function. Loss of Dam and/or Dcm methylation will not make the DNA sensitive to *Eco*K restriction, although loss of K modification will. However, Dam and Dcm modification confer sensitivity to Mrr and Mcr analogues in *Streptomyces* species (MacNeil, 1988).

RECOMBINATION AND ITS EFFECTS ON CLONED DNA INSERTS

During propagation in *E. coli*, DNA inserted into vectors is sometimes rearranged by the proteins involved in DNA recombination. Fortunately, although the genetics and enzymology of recombination in *E. coli* are still not well understood, there are mutant strains available that can provide solutions to two common cloning problems.

Problem 1. The DNA contains dispersed repeated sequences. Recombination occurs between these repeated sequences, causing loss of pieces of the DNA (see sketch 1.4B).

For plasmid libraries, this problem can be solved by propagating the DNA in a *recA*$^-$ host, where homologous recombination does not occur. For libraries made using λ-derived vectors, the vector must also be recombination-defective (*red*). However, only about 30% to 50% of the cells are viable in such a strain, and libraries, particularly phage libraries, may be hard to propagate. Phage λ vectors that are *red* will not make high-titer lysates in *recA* strains, and *red gam* phage will not grow at all, unless the *recBCD* enzyme is also inactivated (see below). Many λ vectors are *red gam* to make use of the Spi$^-$ selection or to make room for larger insert pieces (see *UNIT 1.10*).

Problem 2. The inserted DNA contains closely spaced inverted repeat sequences (palindromes or interrupted palindromes). Such stretches of DNA are not stably propagated in either phage or plasmid vectors. Available knowledge is consistent with the idea that large (>300-bp) palindromes can sometimes form an alternative, hairpin structure that resembles an intermediate found in normal recombination called a Holliday junction, and are then acted upon by the host recombination system in such a way that the hairpin is eliminated or made smaller.

There are strains of bacteria from which phage and plasmid clones containing palindromes are recovered at higher frequency. These bacteria have inactivated exonuclease V (ExoV; encoded by the *recB*, *recC*, and *recD* genes) or the SbcC product (encoded by the *sbcC* gene). Many strains permissive for palindromes have defects in *recB recC* combined with a defect in *sbcA* (which prob-

ably encodes the RecE protein) or *sbcB* (which encodes exonuclease I).

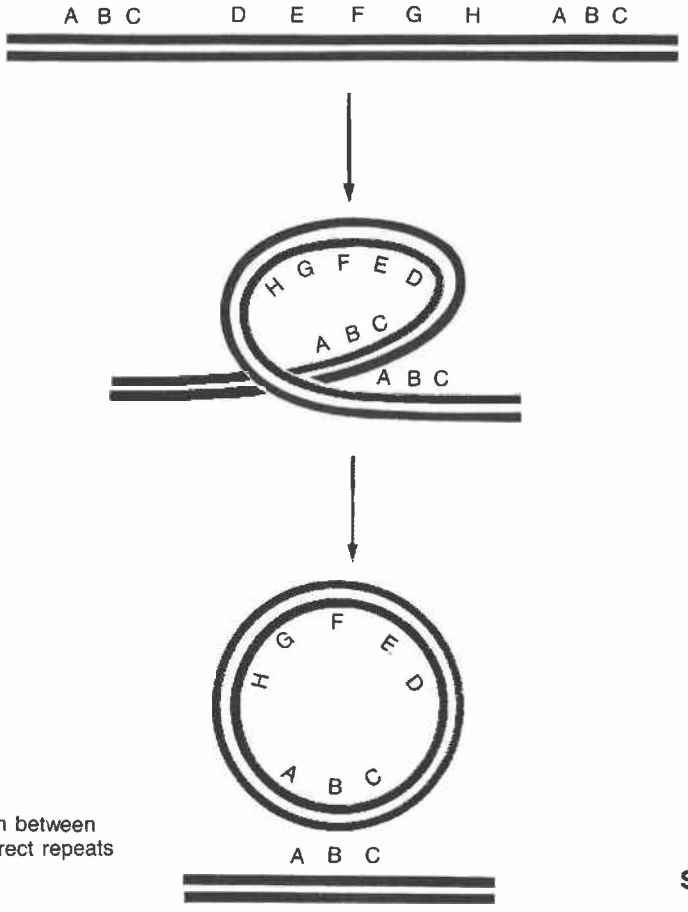
Involvement of ExoV (RecBCD enzyme) in palindrome stabilization was first noticed by Leach and Stahl (1983) using artificially constructed palindromes in λ phages, and hosts mutant in *recB*, *recC*, and *sbcB*. Because *recB recC* strains are sick, they tend to accumulate two additional mutations, one in *sbcB* (the gene for exonuclease I; suppressor of *recBC*), and one in *sbcC*, the biochemical nature of which is unknown (Lloyd and Buckman, 1985). Together these mutations restore recombination and increase cell viability. Wertman et al. (1986) and Wyman et al. (1986) found that both the *recB recC* defect and the *sbcB* defect independently contributed to stabilization of cloned palindromes in λ libraries.

Involvement of *recD* was first investigated by Wertman et al. (1986) and Wyman et al. (1986). *recD* codes for the nuclease activity of ExoV, as distinct from the recombination activity of that enzyme. Strains mutant in *recD* alone are Rec$^+$ and healthy, and are not known to accumulate secondary mutations. Such strains were the best hosts for palindrome stabilization using λ-derived phages in the studies cited above.

The effect of *sbcC* was examined by Chalker et al. (1988), who found that *sbcC* alone was better at maintaining palindromes in λ than *recD* alone and better than *recB recC sbcB*, but that *recD sbcC* strains attained the highest palindrome stability (*recA recD sbcC* strains also maintained the palindrome). A further advantage of *sbcC* mutant strains is that palindromes were maintained in plasmids as well as phage (Chalker et al., 1988), whereas ExoV$^-$ deficient mutants are poor hosts for plasmids regardless of palindrome content (see below).

EFFECTS OF RECOMBINATION-DEFECTIVE STRAINS ON VECTORS

Lambda-derived vectors or clones that are *red gam* (see UNIT 1.10, especially the *spi*$^-$ selection) must be propagated on ExoV$^-$ hosts, because the long linear multimers that are the normal substrate for lambda packaging are exonucleolytically degraded by ExoV in the absence of the Gam protein (Stahl, 1986). These phage will grow well on ExoV$^-$ RecA$^+$ hosts, reasonably well on ExoV$^-$ RecA$^-$ hosts, very poorly on ExoV$^+$ RecA$^+$ (the phage are packaged by an alternative



recombination between homologous direct repeats

Sketch 1.4B

packaging mechanism using circular dimers produced by recombination that depends on *E. coli* proteins), and not at all on ExoV$^+$ RecA$^-$. With ExoV$^-$ RecA$^+$ hosts, clones carrying a recombination hot spot called a Chi site (5′ GCTGGTGG 3′) will outgrow those that don't, resulting in a biased library. The ExoV$^-$ RecA$^+$ host can be *recBC sbcB* or *recD*, and the ExoV$^-$ RecA$^-$ host can be *recB recC sbcB recA* or *recD recA*.

Most cloning plasmids, including ColE1 derivatives like pBR322, and p15A derivatives like the pACYC vectors, are very unstable in ExoV-deficient strains unless selection is maintained. Moreover, they are often unstable or difficult to maintain even with selection. Both *recB recC sbcA* strains (Basset and Kushner, 1984) and *recD* strains (Biek and Cohen, 1986, and references therein) behave this way. However, RecA$^-$ suppresses this effect. Instability is probably due to recombination-initiated rolling-circle replication of the plasmids that leads to synthesis of long linear multimers, which fail to segregate properly at cell division (Silberstein and Cohen, 1987). The problem is particularly severe with very high-copy-number ColE1 derivatives such as pUC vectors, which may be impossible to establish at all in *recD* strains (E.A. Raleigh, unpublished observation).

From the above considerations, a universal host strain for phage and plasmid cloning vectors would have the markers *recA recD sbcC hsdR mcrA mcrB mrr*. Although no such strain has been made, a *recD sbcC hsdR mcrA mcrB* strain, DL491, has been reported recently (Whittaker et al., 1988).

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Definitive review of alpha-complementation.

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Introduction to Plasmid Biology

Bacterial plasmids are self-replicating, circular extrachromosomal DNA molecules. In nature, plasmid varieties occur in exuberant profusion. Natural *E. coli* isolates often carry plasmids specifying resistance to antibiotics, resistance to heavy metals, sensitivity to mutagens, sensitivity or resistance to obscure bacteriophages, production of restriction enzymes, production of rare amino acids, or the catabolism of complicated organic molecules. Replication of these plasmids may or may not require plasmid-encoded proteins and may or may not be synchronized with the cell cycle. Some of these plasmids freely transfer their DNA into other bacterial species, others only transfer their DNA into other *E. coli*, while others do not transfer their DNA at all.

During the 1970s, many plasmids were constructed in the laboratory with fragments of DNA from these naturally occurring plasmids. These artificial plasmids and their derivatives are the most commonly used vectors in recombinant DNA work. All plasmids used as cloning vectors contain three common features: a *replicator*, a *selectable marker*, and a *cloning site*. The replicator is a stretch of DNA that contains the site at which DNA replication begins (usually called the origin of replication, or *ori*) and that also includes genes encoding whatever plasmid-encoded RNAs and proteins are necessary for replication. The selectable marker is usually dominant and is usually a gene encoding resistance to some antibiotic. The cloning site is a restriction endonuclease cleavage site into which foreign DNA can be inserted without interfering with the plasmid's ability to replicate or to confer the selectable phenotype on its host.

UNITS 1.6 & 1.7 describe procedures for making plasmid DNA. The process by which plasmids are introduced into *E. coli* is called *transformation*. Transformation protocols are given in *UNIT 1.8*.

HIGH- AND LOW-COPY-NUMBER REPLICATORS

One way the different plasmid replicators are classified is based on their copy number. Plasmid copy number is usually defined as the number of plasmids per bacterial *cell* grown under some set of standard conditions, but is sometimes used to describe the number of copies of the plasmid per bacterial *chromosome* (cells growing rapidly on rich medium may have 3 to 4 chromosomes, while cells growing slowly on medium which contains a poor carbon source such as succinate usually only average 1.1 chromosomes). This book defines *high-copy-number plasmids* as those which exist in more than 20 copies per bacterial cell grown in liquid LB medium, and *low-copy-number plasmids* as those which exist in fewer than 20 copies per cell. High-copy-number plasmids are used whenever possible in current molecular biological techniques since it is easier to prepare large quantities of pure plasmid DNA from cells that bear them. See Table 1.5.1.

RELAXED AND STRINGENT CONTROL OF COPY NUMBER

High-copy-number plasmids tend to be under *relaxed control*. These plasmids, sometimes called relaxed plasmids, initiate DNA replication in a process controlled by plasmid-encoded functions (see below), which does not depend on the unstable replication initiation proteins synthesized at the start of the bacterial cell cycle. Because their replication does not depend on these unstable host proteins, relaxed plasmids can usually be *amplified*, that is, their copy number can be increased greatly when the cells that bear them are treated with protein synthesis inhibitors such as chloramphenicol or spectinomycin. High-copy-number plasmids usually do not have any mechanism to ensure correct segregation of the plasmid to daughter cells. Low-copy-number plasmids usually are under *stringent control*. Initiation of replication of these plasmids depends on unstable proteins synthesized at the start of the bacterial cell cycle, and thus is synchronized with the replication of the bacterial chromosome. Most plasmids under stringent control contain sites on their DNA called *par* (for partition) loci which in some way enable the plasmid copies to be correctly segregated to daughter cells with very high efficiency.

Table 1.5.1 Characteristics of Commonly Used Plasmid Replicators

Replicator	Prototype plasmid	Size (bp)	Markers on prototype	Copy number	References
pMB1	pBR322	4,362	Amp^r, Tet^r	high; >25	Bolivar et al., 1977
ColE1	pMK16	~4,500	Kan^r, Tet^r, $ColE1^{imm}$	high; >15	Kahn et al., 1979
p15A	pACYC184	~4,000	Eml^r, Tet^r	high; ~15	Chang et al., 1978
pSC101	pLG338	~7,300	Kan^r, Tet^r	low; ~6	Stoker et al., 1982
F	pDF41	~12,800	TrpE	low; 1 to 2	Kahn et al., 1979
R6K	pRK353	~11,100	TrpE	low; <15	Kahn et al., 1979
R1 (R1*drd-17*)	pBEU50	~10,000	Amp^r, Tet^r	low at 30°C; high above 35°C[a]	Uhlin et al., 1983
RK2	pRK2501	~11,100	Kan^r, Tet^r	low; 2 to 4	Kahn et al., 1979
λ *dv*	λ *dvgal*	—[b]	Gal	—	Jackson et al., 1972

[a]Temperature sensitive.
[b]Not known.

MECHANISM OF REPLICATION AND COPY NUMBER CONTROL FOR pMB1-DERIVED AND ColE1-DERIVED CLONING VECTORS

The vast majority of the plasmids used in routine recombinant DNA work contain replicators derived from plasmids pMB1 or ColE1. Cells contain many copies of these plasmids, and although each copy replicates once per cell cycle on the average, some of the copies replicate more than once, and some are not replicated at all. Plasmid replication begins with synthesis of an RNA primer by host RNA polymerase. As the primer transcript is elongated through the *ori* region, the *ori* DNA becomes unpaired and the most recently synthesized part of the transcript pairs with one of the strands. RNase H nicks the primer, and host DNA polymerase I extends the processed primer to begin synthesis of the first strand. Initiation of DNA synthesis is negatively regulated by another plasmid-encoded RNA (called RNA I), which hybridizes to the primer RNA. Pairing of the primer RNA to RNA I is thought to change the secondary structure of the primer RNA in some way that makes it unable to pair with *ori* region DNA. Formation of the RNA I–primer RNA hybrid is somehow facilitated by the action of Rop protein (encoded by the plasmid's *rop* gene). Newer plasmids like pUC plasmids are usually made from pMB1 replicators but do not contain an intact *rop* gene, and are thus maintained in higher copy number than plasmids with intact pMB1 replicons.

PLASMID INCOMPATIBILITY

Cells cannot usually contain two different pMB1- or ColE1-derived plasmids. This fact is an example of a phenomenon called plasmid incompatibility; pMB1- and ColE1-derived plasmids are said to be *incompatible* with one another and to belong to the same *incompatibility group*. Incompatibility for ColE1- and pMB1-derived plasmids is a consequence of two facts: first, that plasmid DNA replication of these plasmids is negatively controlled by RNA I which acts in *trans* on other plasmids with the same primer RNA, and second, that these plasmids lack a mechanism to ensure that each plasmid in a cell replicates once per cell cycle. These two facts ensure that, if a cell contains one pMB1-derived plasmid and is subsequently trans-

formed with another pMB1-derived plasmid, cells selected to contain the second plasmid will usually have lost the first plasmid.

Selectable Markers

Genes encoding proteins which confer resistance to ampicillin (β-lactamases), tetracycline, and chloramphenicol (chloramphenicol acetyltransferases) are the most commonly used selectable markers for plasmid vectors. Typically, cells are transformed with plasmid DNA using the technique described in *UNIT 1.8*, and then plated out on LB plates that contain the proper antibiotic (see recipes in *UNIT 1.4*). These drug-resistance genes are used because they are dominant: the antibiotic-resistance phenotype conferred is dominant to the antibiotic-sensitive phenotype of cells that do not possess them. Other dominant selectable markers that are occasionally used are genes encoding resistance to kanamycin (kanamycin phosphotransferases) or immunity to infection by phage lambda (lambda repressor). Recessive markers are sometimes used in plasmid selections; for example, *leuB*⁻ *E. coli* cannot grow in the absence of leucine, and selection for growth of these strains in the absence of leucine allows isolation of colonies transformed with a plasmid that contains a gene that complements *leuB*.

MAPS OF PLASMIDS

Figures 1.5.1, 1.5.2, 1.5.3, 1.5.4, 1.5.5, and 1.5.6 present maps of plasmids that are in widespread use, or are examples of plasmids whose special functions make them useful for particular techniques described in this manual.

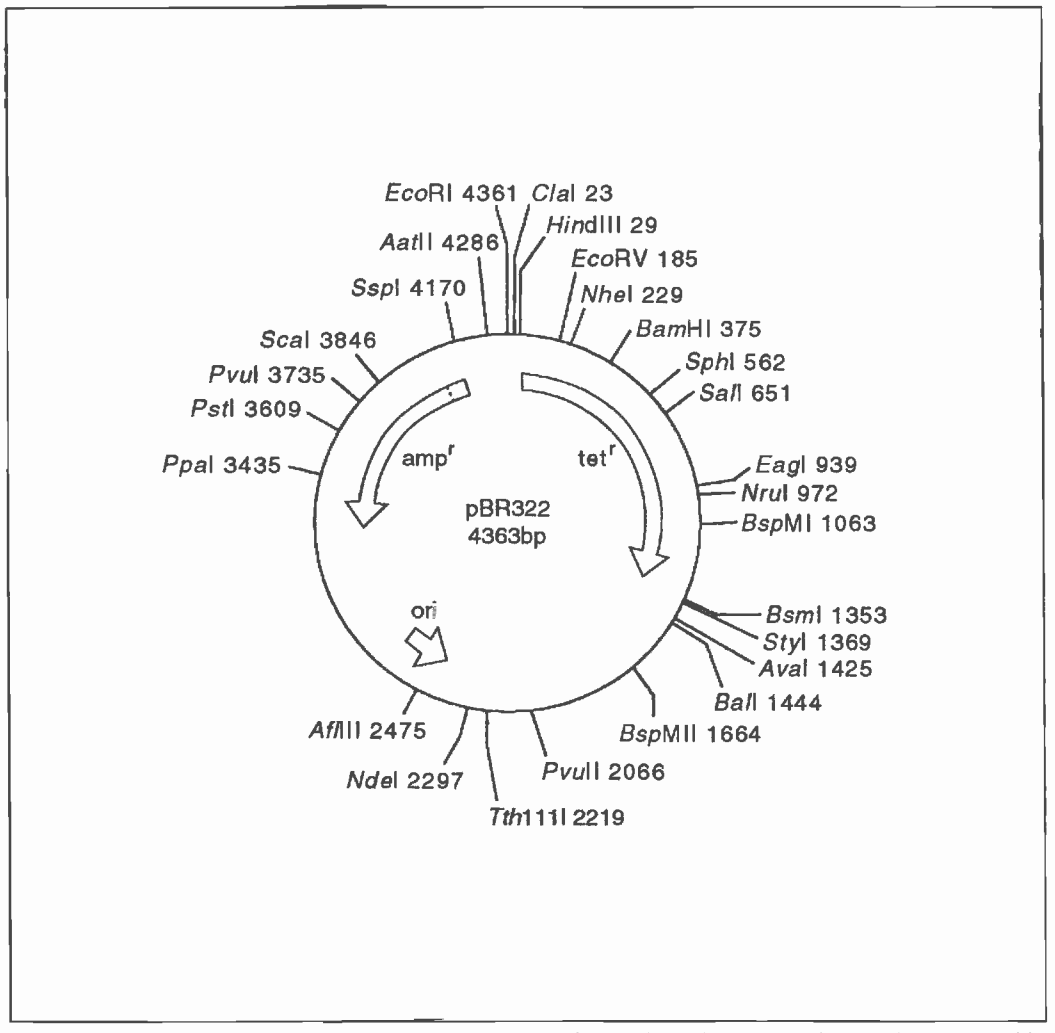

Figure 1.5.1 pBR322. pBR322 is a very commonly used cloning vector. It contains an amplifiable pMB1 replicator and genes encoding resistance to ampicillin and tetracycline. Insertion of DNA into a restriction site in either drug-resistance gene usually inactivates it and allows colonies bearing plasmids with such insertions to be identified by their inability to grow on medium with that antibiotic (Bolivar et al., 1977; sequence in Sutcliffe, 1978).

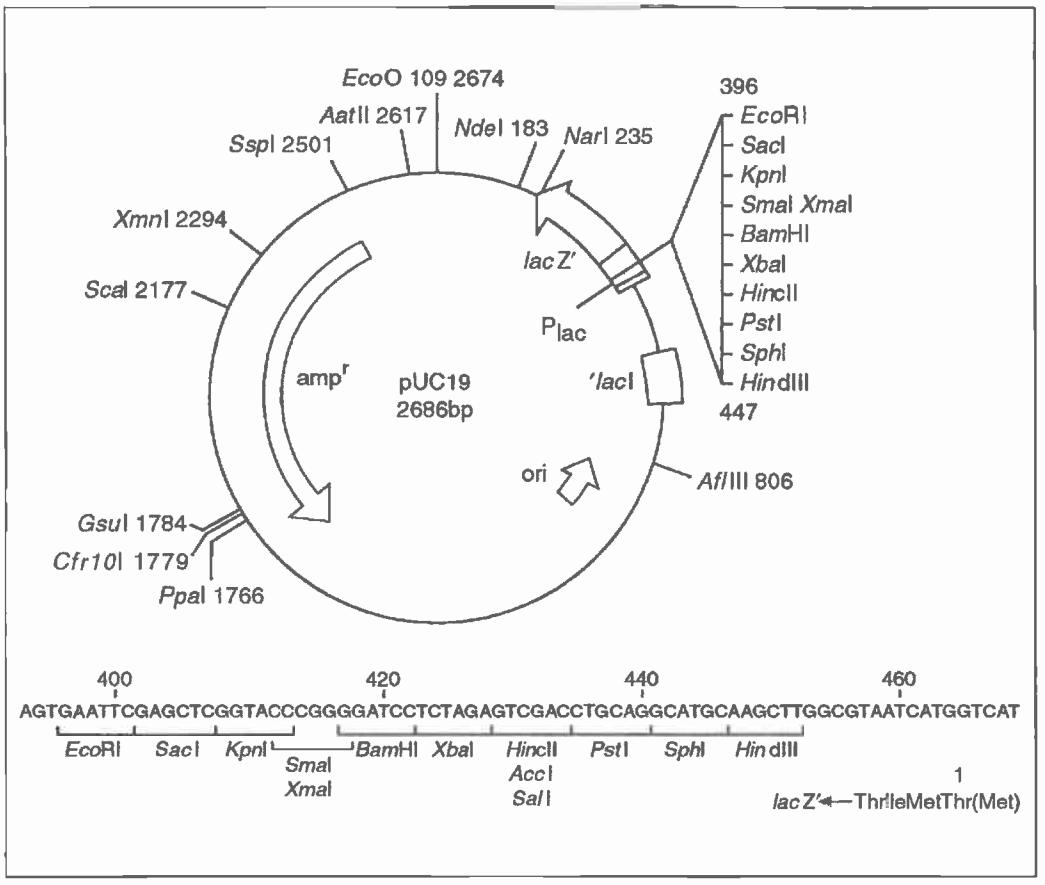

Figure 1.5.2 pUC19. pUC19 belongs to a family of plasmid vectors that contain a polylinker inserted within the alpha region of the *lacZ* gene. The polylinkers are the same as those used in the M13mp series and their sequence is given in Figure 1.14.2. pUC19 and pUC18 have the same polylinker but in opposite orientations. Under appropriate conditions (see *UNIT 1.4* for a description), colonies that bear plasmids containing a fragment inserted into the polylinker form white colonies instead of blue ones. These pMB1-derived plasmids maintain a high copy number because they lack an intact *rop* gene (see earlier this unit); moreover, they are thought to bear another mutation in the *ori* region that increases the copy number. Wild-type and recombinant plasmids confer ampicillin resistance and can be amplified with chloramphenicol (Norrander et al., 1983). In addition wild-type plasmids confer a $LacZ^+$ phenotype to appropriate cells (e.g., JM101 cells, *UNIT 1.4*).

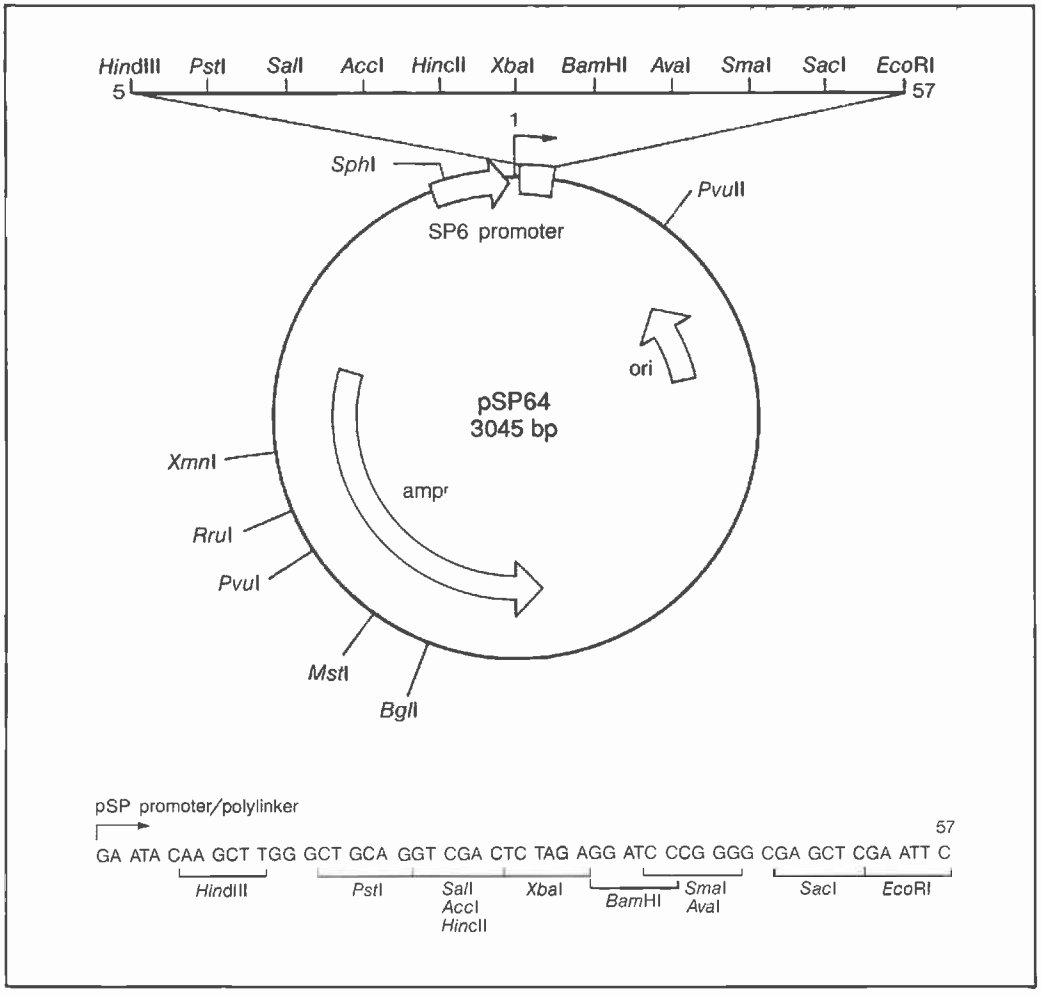

Figure 1.5.3 pSP64. pSP64 is an example of a plasmid vector that contains promoters recognized by efficient phage RNA polymerases. The promoter of pSP64 is recognized by bacteriophage SP6 RNA polymerase. Transcription from this promoter reads into a polylinker that is oriented oppositely in pSP64 and pSP65. When these vectors are used, DNA is inserted into the polylinker and the vector is linearized by cutting at a site downstream of the inserted DNA. SP6 polymerase and ribonucleoside triphosphates are added, so that one strand of the DNA inserted into the polylinker is copied into RNA. The ability to generate large amounts of single-stranded RNA is useful for many procedures, for example in mapping the ends of RNAs in the procedure given in *UNIT 4.7*. SP6 vectors and their use are described in Zinn et al. (1983) and Melton et al. (1984).

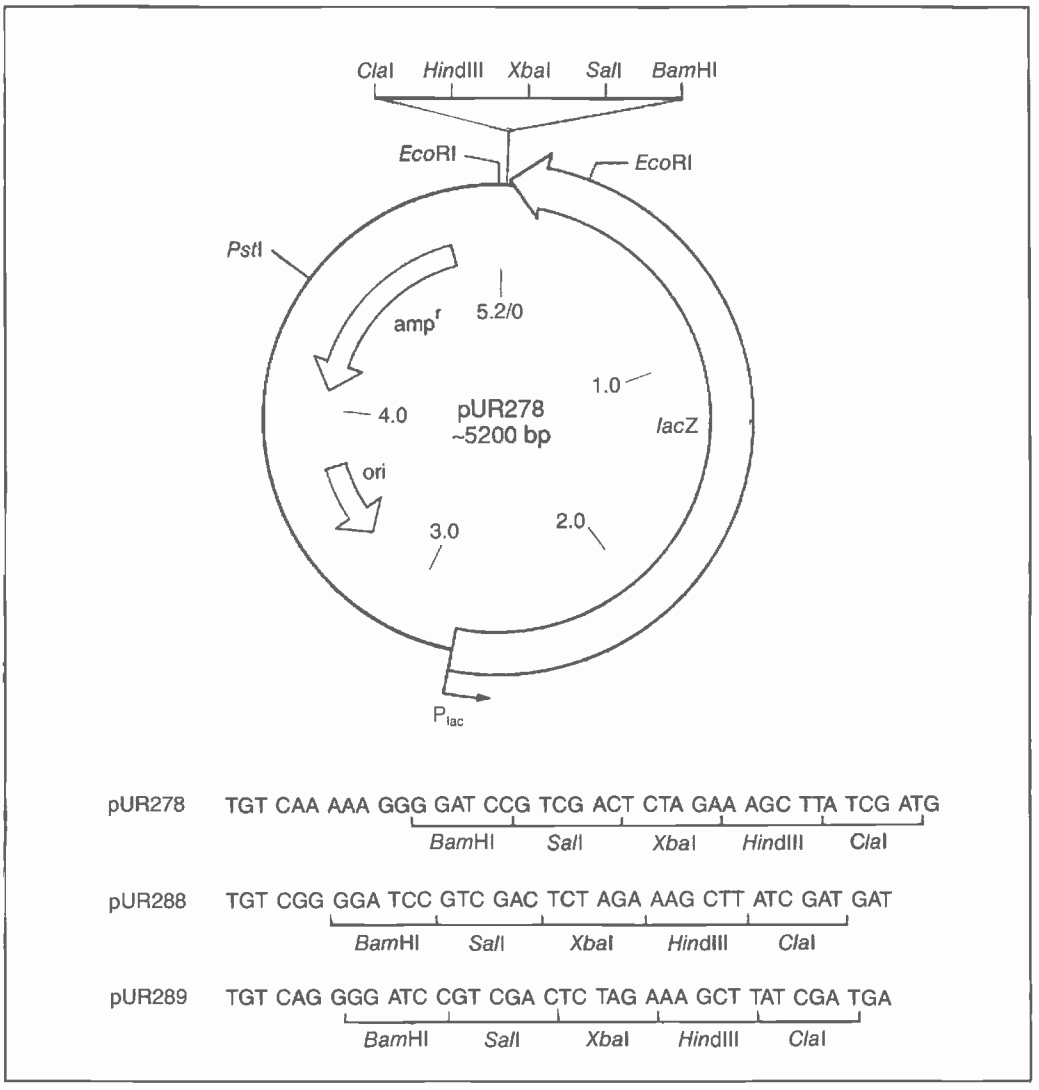

Figure 1.5.4 pUR278. pUR278 is an example of a fusion-protein expression vector. It is a member of a family of plasmid vectors that contain the *lacZ* gene transcribed by the *lac* UV5 promoter. In a *lacI*^{q} strain, transcription of the *lacZ* gene from the UV5 promoter is repressed, but is derepressed when IPTG is added to the growth medium. DNA fragments that contain coding sequences can be inserted into the polylinker located at the end of the *lacZ* gene. Derepression of the UV5 promoter causes expression of the resulting fusion protein, which can then often be easily purified (Rther and Miller-Hill, 1983).

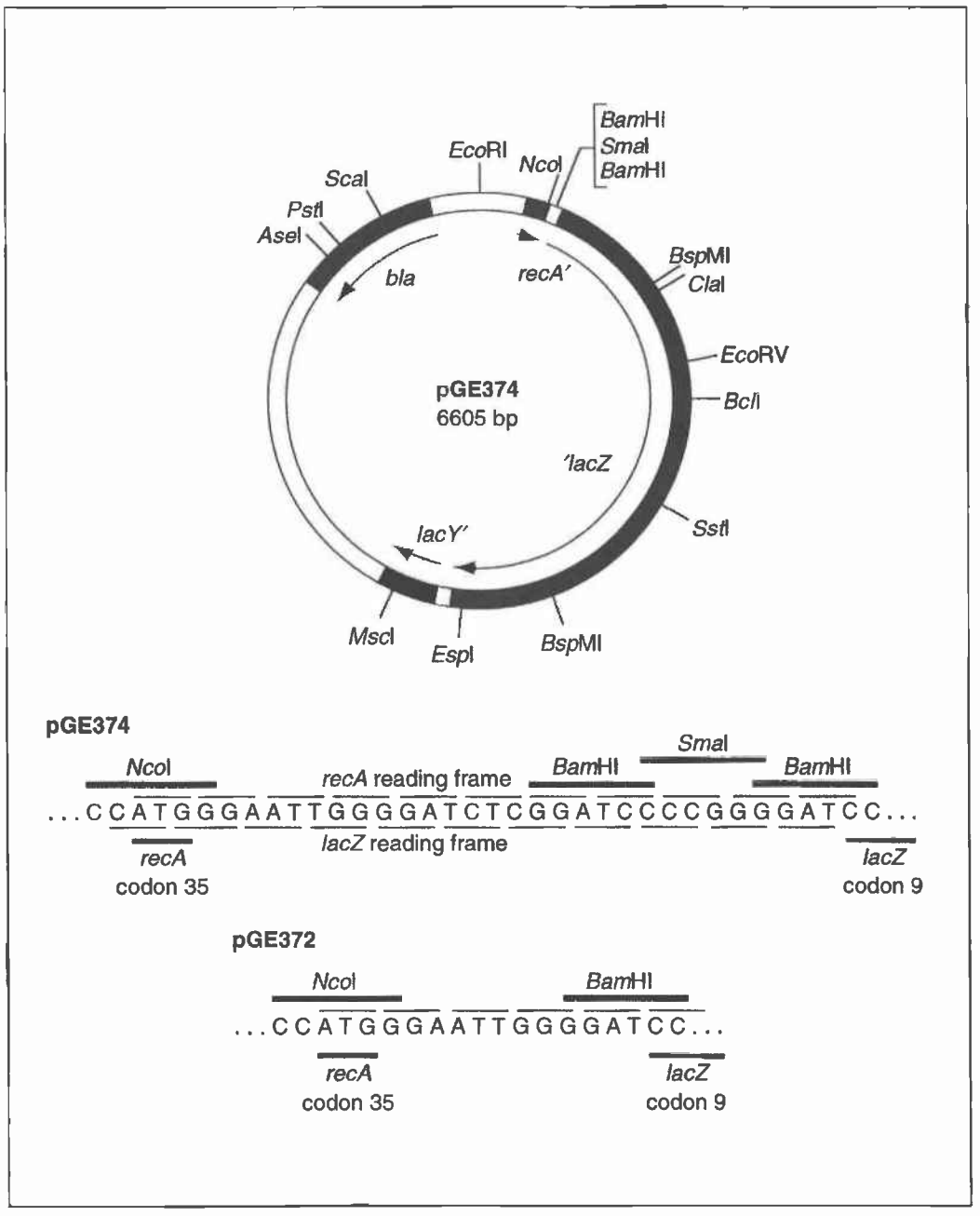

Figure 1.5.5 **pGE374.** pGE374 is an example of a vector used to clone and express open reading frames (ORFs). This expression system operates on the same principle as vectors that use the *ompF* regulatory region (described by Weinstock et al., 1983); however, the *recA*-carrying vector pGE374 is better behaved and its expression system is more reliable than that of the *ompF* vector system. pGE374 confers a $LacZ^-$ phenotype unless an ORF has been inserted that realigns *recA* and lacZ and creates a $LacZ^+$ phenotype. The basal level of expression of a single-copy *recA* gene is about 1000 molecules per cell, so it is not necessary to induce strains carrying pGE374 with inserts in order to detect their LacZ phenotype. The basal level of expression is sufficient for detection of LacZ in plasmid-carrying strains grown on Xgal indicator plates. The structures of the *recA/lacZ* junctions in pGE374 and its relative pGE372 are shown below the vector. In pGE374 the *recA* and *lacZ* coding sequences are out of frame so that no hybrid protein is produced. Plasmid pGE372 is similar, except that the *recA* and *lacZ* sequences are in frame and hybrid protein is produced.

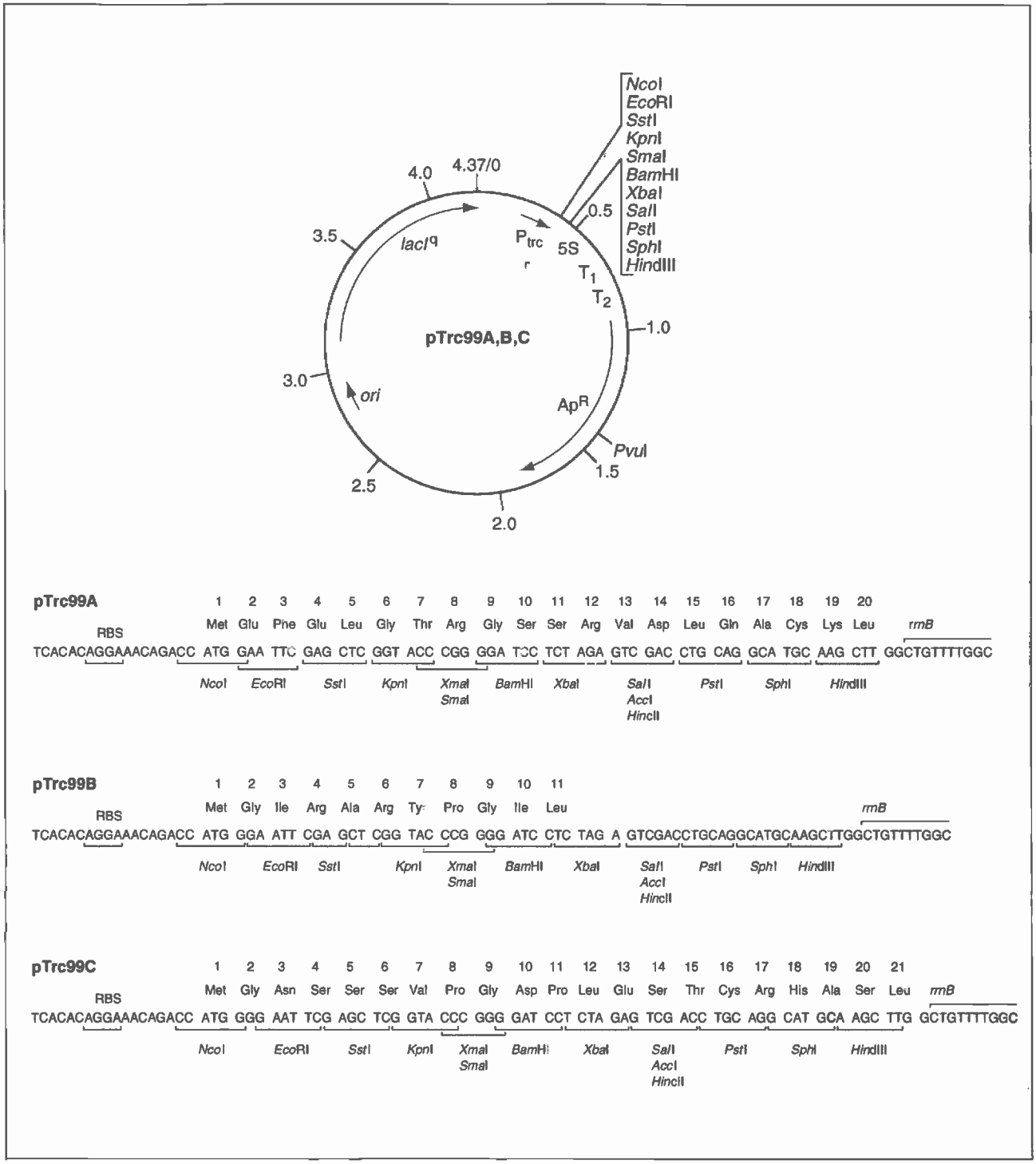

Figure 1.5.6 pTrc 99A,B,C. The pTrc series of plasmid expression vectors facilitates the regulated expression of genes in *E. coli*. These vectors carry the strong hybrid *trp/lac* promoter, the *lacZ* ribosome binding site (RBS), the multiple cloning site of pUC18 that allows insertion in three reading frames, and the *rrnB* transcription terminators (see polylinker sequences given below the vector diagram). These vectors are equally useful for the expression of non-fused proteins (resulting from insertion into the *Nco*I site) or for expression of fusion proteins (using one of the cloning sites in the correct translational frame).The presence of the *lacI*^{q} allele on the plasmid ensures complete repression of the hybrid *trp/lac* promoter during cloning and growth in any host strain (see Amann et al., 1988, for further details).

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Minipreps of Plasmid DNA

Although there are a large number of protocols for the isolation of small quantities of plasmid DNA from bacterial cells (minipreps), this unit presents four procedures based on their speed and success: the alkaline lysis prep, a modification of the alkaline lysis prep that is performed in 1.5-ml tubes or 96-well microtiter dishes, the boiling method, and a lithium-based procedure. A support protocol provides information on storing plasmid DNA.

ALKALINE LYSIS MINIPREP

BASIC PROTOCOL

The alkaline lysis procedure (Birnboim and Doly, 1979, and Birnboim, 1983) is the most commonly used miniprep. Plasmid DNA is prepared from small amounts of many different cultures (1 to 24) of plasmid-containing bacteria. Bacteria are lysed by treatment with a solution containing sodium dodecyl sulfate (SDS) and NaOH (SDS denatures bacterial proteins, and NaOH denatures chromosomal and plasmid DNA). The mixture is neutralized with potassium acetate, causing the covalently closed plasmid DNA to reanneal rapidly. Most of the chromosomal DNA and bacterial proteins precipitate—as does the SDS, which forms a complex with potassium—and are removed by centrifugation. The reannealed plasmid DNA from the supernatant is then concentrated by ethanol precipitation.

Materials

LB medium (*UNIT 1.1*) containing appropriate antibiotic (Table 1.4.1)
Glucose/Tris/EDTA (GTE) solution
TE buffer (*APPENDIX 2*)
NaOH/SDS solution
Potassium acetate solution
95% and 70% ethanol
10 mg/ml DNase-free RNase (optional; *UNIT 3.13*)
1.5-ml disposable microcentrifuge tubes

1. Inoculate 5 ml sterile LB medium with a single bacterial colony. Grow to saturation (overnight).
2. Spin 1.5 ml of cells 20 sec in a microcentrifuge at maximum speed to pellet. Remove the supernatant with a Pasteur pipet.

   The spins in steps 2 and 6 can be performed at 4°C or at room temperature. Longer spins make it difficult to resuspend cells.

3. Resuspend pellet in 100 µl GTE solution and let sit 5 min at room temperature.

   Be sure cells are completely resuspended.

4. Add 200 µl NaOH/SDS solution, mix by tapping tube with finger, and place on ice for 5 min.
5. Add 150 µl potassium acetate solution and vortex at maximum speed for 2 sec to mix. Place on ice for 5 min.

   Be sure mixing is complete.

6. Spin 3 min as in step 2 to pellet cell debris and chromosomal DNA.
7. Transfer supernatant to a fresh tube, mix it with 0.8 ml of 95% ethanol, and let sit 2 min at room temperature to precipitate nucleic acids.
8. Spin 1 min at room temperature to pellet plasmid DNA and RNA.

9. Remove supernatant, wash the pellet with 1 ml of 70% ethanol, and dry pellet under vacuum.

10. Resuspend the pellet in 30 µl TE buffer and store as in support protocol. Use 2.5 to 5 µl of the resuspended DNA for a restriction digest.

Contaminating RNA may interfere with detection of DNA fragments on the agarose gel; it can be destroyed by adding 1 µl of a 10 mg/ml RNase solution (DNase-free) to the digestion mixture.

ALTERNATE PROTOCOL

ALKALINE LYSIS IN 96-WELL MICROTITER DISHES

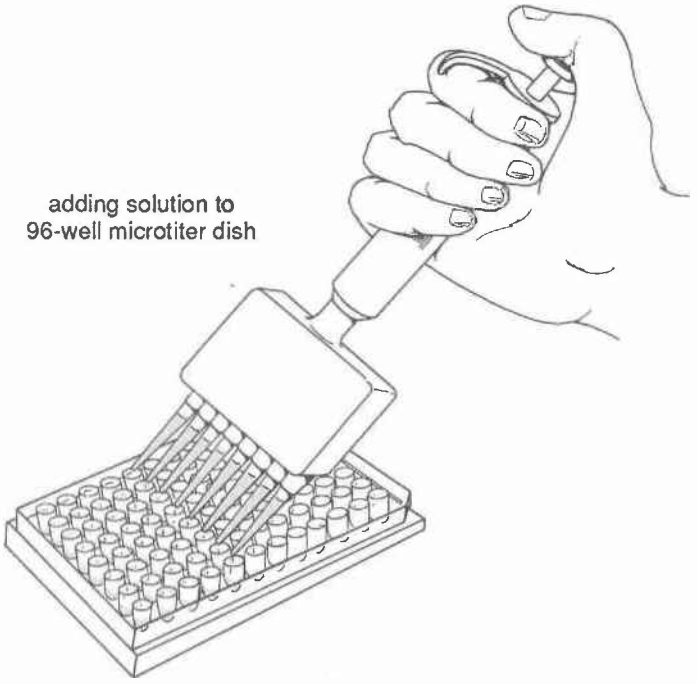
Escherichia coli cells that contain plasmids are grown and lysed, and the plasmid DNA is precipitated—all in the wells of 96-well microtiter dishes. This procedure makes it possible to perform hundreds of rapid plasmid preps in a day. It is based on an unpublished procedure by Brian Seed of Massachusetts General Hospital.

Additional Materials

TYGPN medium (*UNIT 1.1*)
70% ethanol, ice-cold
Isopropanol
96-well microtiter plates (Dynatech PS plates or equivalent)
Multichannel pipetting device (8-prong Costar; 12-prong Titer Tek)
Multitube vortexer
Sorvall RT-6000 low-speed centrifuge, or equivalent, with microplate carrier in H-1000B rotor

1. Add 0.3 ml sterile TYGPN medium to each well of a 96-well microtiter plate (see sketch 1.6A). Inoculate each well with a single plasmid-containing colony.

The 96-well microtiter plates used must have U-shaped bottoms. To take full advantage of this protocol, one should perform all pipetting steps with a multichannel pipetting device.



Sketch 1.6A

2. Grow bacteria to saturation at 37°C (~48 hr).

   All subsequent steps are performed at room temperature unless otherwise noted.

   Potassium nitrate in the TYGPN medium presumably acts as a terminal electron acceptor when the bacteria in the wells are growing anaerobically, resulting in high cell densities.

3. Spin saturated cultures in H-1000B rotor with microplate carrier for 10 min at 2000 rpm (600 × *g*), 4°C. Decant supernatant with brief flick.

   A microplate carrier is available for the Beckman JS-4.2 rotor. The same rpm values can be used for the JS-4.2 as those given here for the Sorvall H-1000B (see APPENDIX 1 for rotor conversion values).

4. Resuspend cells in the well bottoms by clamping the plate in a multitube vortexer and running it 20 sec at setting 4.

5. Add 50 µl GTE solution to each well.

6. Add 100 µl NaOH/SDS solution to each well. Wait 2 min.

7. Add 50 µl potassium acetate solution to each well.

8. Cover with plate tape or parafilm. Agitate vigorously in vortexer 20 sec at setting 4. Spin 5 min at 2000 rpm (600 × *g*), 4°C.

9. Insert a pipet tip just at the edge of the U in the bottom of the well. Remove 200 µl from each well and transfer to a new plate.

   Do not try to recover all the fluid in each well.

10. Add 150 µl isopropanol to each well of the new plate. Cover with plate tape, agitate, and chill 30 min at –20°C.

11. Spin 25 min at 2000 rpm, 4°C, and decant supernatant. Wash pellets with cold 70% ethanol, gently decant supernatant, wash with 95% ethanol, and again gently decant supernatant.

    Pellets often shrink visibly during the 70% ethanol wash, as impurities in them are dissolved.

    Restriction enzymes will not cut well if the DNA is contaminated with even very small amounts of NaOH/SDS or potassium acetate solutions. It is therefore very important to decant the supernatants from the isopropanol precipitation and ethanol washes thoroughly, but not so vigorously that the pellets are flung out of the well bottoms.

    If pellets become detached during either washing step, the plate should be respun at 2500 rpm for 5 min to bring the pellets to the bottom of the wells again.

12. Air dry pellets for 30 min, then resuspend in 50 µl TE buffer. Store as in support protocol and use 10-µl aliquots for digestion.

BASIC PROTOCOL

BOILING MINIPREP

Bacteria that contain plasmid DNA are broken open by treatment with lysozyme, Triton (a nonionic detergent), and heat. The chromosomal DNA remains attached to the bacterial membrane and is pelleted to the bottom of a centrifuge tube during a brief spin. Plasmid DNA is precipitated from the supernatant with isopropanol (Holmes and Quigley, 1981). This procedure is recommended for preparing small amounts of plasmid DNA from 1 to 24 cultures. It is extremely quick, but the quality of DNA produced is lower than that from the alkaline lysis miniprep.

Materials

LB medium (*UNIT 1.1*) containing appropriate antibiotic (Table 1.4.1)
STET solution
Hen egg white lysozyme
Isopropanol, ice-cold
TE buffer (*APPENDIX 2*)
10 mg/ml DNase-free RNase (optional; *UNIT 3.13*)
1.5-ml disposable microcentrifuge tubes
Boiling water bath (100°C)

1. Inoculate 5 ml sterile LB medium with a single bacterial colony. Grow at 37°C at least until mid-log phase ~6 hr, (a freshly saturated overnight culture works even better; see *UNIT 1.2*).

2. Transfer 1.5 ml of the saturated culture to a 1.5-ml microcentrifuge tube and pellet the cells by spinning 20 sec in microcentrifuge at maximum speed. Discard supernatant with a Pasteur pipet.

   The spins in steps 2, 6, and 7 can be performed at 4°C or room temperature. Longer spins make it difficult to resuspend cells.

3. Resuspend the bacteria in 300 µl of STET solution containing 200 µg lysozyme. Vortex to achieve complete suspension.

   Be sure cells are completely resuspended in order to maximize the number of cells exposed to the lysozyme and consequently the yield of plasmid DNA.

4. Place tube on ice for 30 sec to 10 min.

   The time required for this step can vary between the limits indicated without affecting the yield or quality of the plasmid DNA.

5. Place tube in a boiling water bath (100°C) 1 to 2 min.

   Heat and detergents cause the weakened cell walls to break, releasing plasmid DNA and RNA, but not the larger bacterial chromosome which remains attached to or trapped inside the lysed cells.

6. Spin in microcentrifuge 15 to 30 min at maximum speed.

   The pellet, which should be fairly gummy, contains bacterial debris as well as chromosomal DNA. The supernatant contains plasmid DNA and RNA.

7. Pipet off supernatant into a new tube, carefully, without dislodging pellet. Mix with 200 µl (an equal volume) of cold isopropanol. Place at –20°C for 15 to 30 min. Spin 5 min in microcentrifuge at maximum speed.

   The cold isopropanol precipitates the plasmid DNA and cellular RNA. Considerably shorter incubation periods (e.g., 2 to 5 min) may be sufficient for precipitation.

8. Remove the supernatant by inverting the tube and flicking it several times. Dry the

pellet by placing under a vacuum until it looks flaky.

If a vacuum source is unavailable, the pellet can be air dried.

9. Resuspend the pellet in 50 µl TE buffer and store as in the support protocol. Use 5 µl of the resuspended DNA for a restriction digest.

   Contaminating RNA may interfere with detection of DNA fragments on the agarose gel; it can be destroyed by adding 1 µl of a 10 mg/ml RNase solution (DNase-free) to the digestion mixture.

LITHIUM MINIPREP

BASIC PROTOCOL

Plasmid DNA is obtained from *E. coli* grown on plates as colonies or in liquid cultures. Bacterial cells harboring plasmid DNA are sequentially treated with Triton X-100/LiCl and phenol/chloroform. These steps solubilize plasmid DNA while precipitating chromosomal DNA with cellular debris. The debris is removed by centrifugation. This isolation procedure yields preparations of plasmid DNA that are virtually devoid of chromosomal DNA.

The procedure described here (originally presented by He et al., 1990) for small-scale isolation of plasmid DNA can also be readily extended for large-scale preparations as described in the annotation to the final step. The merit of the approach is that it is extremely reliable and rapid—requiring no more than 20 min of simple and economical operations for a preparation. The final plasmid DNA preparations are of a purity and quality usable for most biological applications.

Materials

TELT solution
LB medium (*UNIT 1.1*) containing appropriate antibiotic (Table 1.4.1)
1:1 (w/v) phenol/chloroform (*UNIT 2.1*)
100% ethanol, prechilled to –20°C
TE buffer (*APPENDIX 2*)
10 mg/ml DNase-free RNase A (optional; *UNIT 3.13*)
1.5-ml disposable microcentrifuge tubes

NOTE: All steps are performed at room temperature.

1. To isolate plasmid DNA from transformant colonies grown on agar plates, prepare the cells as follows:

   a. Using a microspatula, scoop out an entire bacterial colony grown to 2- to 5-mm diameter on an LB agar plate. Transfer the colony to a 1.5-ml microcentrifuge tube containing 100 µl TELT solution.

   b. Vortex thoroughly to suspend the cells. Proceed to step 3.

2. To isolate plasmid DNA from liquid cultures, prepare the cells as follows:

   a. Inoculate a colony of bacteria into 1.8 ml of sterile LB medium supplemented with appropriate antibiotic. Grow to saturation with shaking for 18 to 24 hr at 30°C (see *UNIT 1.2*).

      Glass test tubes with plastic caps are suitable. Place the tubes at a suitably inclined angle to achieve good agitation.

   b. Carefully transfer the entire culture volume into a 1.5-ml microcentrifuge tube.

      At this stage the volume of the liquid culture will have been reduced to ~1.5 ml.

c. Pellet the cells by spinning in a microcentrifuge (10,000 × *g*) for 20 sec.

The spins in steps 2, 4, 7, and 9 can be performed at 4°C or room temperature. Centrifugation for longer periods or at higher speeds makes it difficult to resuspend the cells in the following step.

d. With the tube held in a vertical position, aspirate the supernatant using a long-tipped Pasteur pipet connected to a vacuum line.

e. Add 100 µl of TELT solution to the pellet and resuspend by vortexing.

Ensure that the cells are thoroughly suspended. See annotation to step 11 for scaled-up DNA preparations.

3. Add 100 µl of 1:1 phenol/chloroform and thoroughly vortex for 5 sec.

This mixture may be left at room temperature for ≤15 min. Plasmid yield will elevate with increasing duration of incubation; however, incubation periods >15 min may result in phenol-mediated modification of DNA.

4. Microcentrifuge 1 min at 15,000 × *g* (maximum speed).

5. Using a pipettor or similar device, carefully withdraw 75 µl of the upper aqueous phase and transfer the contents into a clean microcentrifuge tube.

Do not agitate the resolved phases. If mixing occurs, recentrifuge. When collecting the top layer avoid picking the debris at the interface.

6. To the supernatant, add 150 µl of chilled 100% ethanol. Mix the contents well to precipitate the plasmid DNA.

7. Pellet the nucleic acids by microcentrifuging 5 min at maximum speed.

8. Discard the supernatant by inverting the tube. When all the supernatant has drained, hold the tube in the same position for a few seconds and wipe off the last droplet from the rim of the tube by touching the edge of a Kleenex paper tissue.

9. Wash the pellet with 1 ml of cold 100% ethanol and harvest the nucleic acid pellet as in steps 6 and 7.

After centrifugation, decant the supernatant carefully as the pellet may be loose.

10. Cap the tube. Stab a small hole in the cap with a thumbtack or a syringe needle. Place the tube in a vacuum dessicator (without dessicant). Apply vacuum until the nucleic acid pellet appears completely dry.

A water-pumped vacuum line suffices for the purpose and usually takes ≤15 min.

11. Dissolve the pellet in 30 µl TE buffer. Vortex the contents well, capturing most of the DNA around the inner surface of the microcentrifuge tube. Store as in the support protocol and use 2 to 5 µl of DNA solution in a final 20-µl reaction volume for restriction digestion.

Contaminating RNA may interfere with detection of DNA fragments on the agarose gel; it can be destroyed by adding 1 µl of a 10 mg/ml RNase solution (DNase-free) to the digestion mixture.

For scaled-up plasmid DNA preparations (He et al., 1991), increase the amounts of TELT solution and 1:1 phenol/chloroform in direct proportion to the culture volume used. For cultures ≤5 ml, transfer the cells after suspension in TELT buffer into a microcentrifuge tube. Wash the final nucleic acids pellet twice with 1 ml of 100% ethanol. For cultures between 5 and 100 ml, use Corex glass tubes for treatment with TELT and phenol/chloroform and for centrifugations (Sorvall RC-5C centrifuge at 6000 × g).

STORAGE OF PLASMID DNA

SUPPORT PROTOCOL

Plasmids can be maintained for a short period (up to 1 month) in bacterial strains simply by growing on selective plates and storing at 4°C. For permanent storage, bacteria harboring the plasmid should be grown to saturation in the presence of the appropriate selective agent. An equal volume (~1 to 2 ml) of bacteria should be added to sterile 100% glycerol or a DMSO-based solution (recipe in *UNIT 1.3*) and frozen at –70°C in sterile vials. Cells taken from storage should again be grown on a selective plate (*UNIT 1.1*), and the plasmid DNA should be checked by restriction analysis (*UNIT 3.1*).

Plasmid DNA can be stored in TE buffer at 4°C for several weeks or preserved for several years by storing at –20° or –70°C. Most investigators prefer to store plasmids as frozen DNA, due to the widely held belief that plasmids stored in bacteria are sometimes lost, are rearranged, or accumulate insertion sequences and transposons during storage or on revival. Although such rearrangements certainly occur during storage of plasmids in bacteria in stab vials, we are unaware of any report of rearrangements affecting plasmids stored in frozen cells.

REAGENTS AND SOLUTIONS

Glucose/Tris/EDTA (GTE) solution

50 mM glucose
25 mM Tris·Cl, pH 8.0
10 mM EDTA
Autoclave and store at 4°C

NaOH/SDS solution

0.2 N NaOH
1% (wt/vol) sodium dodecyl sulfate (SDS)
Prepare immediately before use

5 M potassium acetate solution, pH 4.8

29.5 ml glacial acetic acid
KOH pellets to pH 4.8 (several)
H_2O to 100 ml
Store at room temperature (do not autoclave)

STET solution

8% (wt/vol) sucrose
5% (wt/vol) Triton X-100
50 mM EDTA
50 mM Tris·Cl, pH 8.0
Filter sterilize and store at 4°C

TELT solution

2.5 M LiCl
50 mM Tris·Cl, pH 8.0
62.5 mM Na_2 EDTA
4% (wt/vol) Triton X-100
Store as 1- to 5-ml aliquots frozen at –20°C (do not filter sterilize or autoclave)

COMMENTARY

Background Information

Isolation of small quantities of plasmid DNA from bacterial cells is essential for the analysis of recombinant clones (*UNITS 3.2 & 6.3*). A myriad of plasmid DNA miniprep methods now exist and investigators are generally remarkably loyal to his or her own particular protocol. This unit presents three of the most widely used and reliable methods—alkaline lysis, the boiling method, and a lithium-based miniprep. The success of any of these procedures is largely a function of the expertise of the investigator; choice of method is therefore determined by personal preference as well as the size and type of the plasmid and the host strain of *E. coli*. With practice, all three protocols yield plasmid DNA of sufficient quantity and quality for use in most enzymatic manipulations (Chapters 3 & 7), and in most bacterial (*UNIT 1.8*) and yeast (*UNIT 13.7*) transformation procedures.

The procedures presented in this unit allow for the preferential recovery of circular plasmid DNA over linear chromosomal DNA. Treatments with either base or detergent (used in all three procedures) disrupt base pairing and cause the linear chromosomal DNA to denature and separate. In contrast, because of its supercoiled configuration, covalently closed circular plasmid DNA is unable to separate and readily reforms a correctly paired superhelical structure under renaturing conditions.

In the alkaline lysis miniprep, treatment with SDS and NaOH breaks open bacterial cells. Subsequent addition of potassium acetate preferentially reanneals covalently closed plasmid DNA, while chromosomal DNA and proteins are trapped in a complex formed between the potassium and SDS. The lysis treatment in the boiling miniprep causes chromosomal DNA to remain attached to the bacterial membrane, while plasmid DNA remains in the supernatant. In the lithium method, treatment of bacterial cells with Triton X-100/LiCl results in dissolution of the inner bacterial plasma membrane. However, this treatment has no effect on the overall morphology of the cells as observed microscopically, nor does it lead to release of plasmid DNA from the cells. Subsequent addition of phenol/chloroform leads to denaturation and precipitation of intracellular proteins. The concomitant rapid shrinkage of the cells preferentially expels soluble, supercoiled plasmid DNA into the medium while retaining chromosomal DNA and denatured cellular protein with the bulk of the cell mass. Bacterial morphology, particularly of the cell wall envelope, is preserved under these conditions for at least 30 min, at which point cell lysis ensues. In all preparations, chromosomal DNA is removed with cellular debris by centrifugation, and soluble, supercoiled plasmid DNA is concentrated by ethanol precipitation.

In general, all three methods provide plasmid DNA of comparable yield and quality suitable for most biological applications. Yield of DNA is determined more by the type of plasmid than by method of isolation. Plasmids derived from pBR322 generally give lower (but, for most applications, sufficient) yields compared with the more recently derived pUC-like vectors, which contain a lesion in the plasmid-encoded *rop* gene, causing the plasmid to be maintained in high copy number in the cell (see *UNIT 1.5*).

All three methods are successful for the isolation of small (<10-kb) plasmids. Larger plasmids are generally poorly isolated using the lithium method, with yields <10% of those in the pUC-size range. These larger-sized plasmids may be retained along with chromosomal DNA in the mesh-like structure of the cell wall. In rare instances this problem may extend to plasmids that exist in low copy number and to those contained in strains with unusual cell wall compositions (see discussion of strain considerations below). The alkaline prep is better suited for efficient isolation of large-sized or low-copy-number plasmids (see critical parameters).

Strain background is also a consideration when selecting a miniprep procedure. When lysed with detergent or heat (as in the boiling or lithium procedures), strains such as HB101 and its derivatives may release a large amount of carbohydrate that contaminates plasmid DNA and inhibits many restriction endonucleases. In addition, HB101-related strains express endonuclease A which, if not inactivated, may degrade plasmid DNA in the presence of magnesium (during restriction enzyme digestion). Plasmids harbored in these strains may be better isolated using the alkaline lysis method.

In general, the alkaline lysis miniprep can be used for a variety of plasmid types and

sizes carried in most host strains. The boiling method is perhaps the most forgiving of the three, and the lithium miniprep, although restricted for use with small plasmids in certain hosts, is the most rapid method.

Critical Parameters and Troubleshooting

The successful isolation of small quantities of DNA from bacterial cells, regardless of the protocol, is largely dependent on the strain of *E. coli* used. For example, DNA isolated from strains C600, DH1 and LE392 is of good quality, while DNA isolated from strains HB101 and the JM100 series is of lesser quality (see Table 1.4.5). The latter strains have high nuclease activity (endonuclease A, which is not completely inactivated by boiling), necessitating further purification with a phenol extraction (*UNIT 2.1*) or an additional precipitation with ammonium acetate (*UNIT 2.1*). If one protocol fails to yield DNA with a particular strain of *E. coli*, it would be best to try an alternative method. The alkaline lysis miniprep seems to be the most consistent regardless of strain used, while the lithium method incorporates a phenol extraction and may be well suited for isolation of plasmid from strains with high nuclease activity.

Failure to isolate DNA using any of the protocols could be due to DNase contamination of the RNase. This problem can be circumvented by leaving out or using considerably less RNase. Other procedure-specific guidelines are described below.

Alkaline lysis miniprep. The DNA yield can generally be increased by adding 500 mg/ml of lysozyme to the 100-µl suspension of cells in the glucose/Tris/EDTA (GTE) solution (step 3).

With a few variations, this protocol can also be used to isolate low-copy-number plasmids (*UNIT 1.5*), as follows: Increase the number of starting cells to 3 ml and include lysozyme in the GTE solution as described above. Because yields are lower with strains harboring low-copy-number plasmids, it will be necessary to use at least 5 µl of DNA for a restriction digest.

If the isolated DNA fails to cut with restriction endonucleases, the most common cause is inadequate washing of the pellets after the ethanol precipitation step. Precipitating the DNA a second time with ethanol, or washing the pellets from the first precipitation with 70% ethanol, will usually clean up the DNA enough for restriction enzyme cutting.

Boiling miniprep. Failure to isolate DNA is sometimes caused by incomplete cell lysis. If this appears to be the case, try using a new bottle of lysozyme powder. Apparent failure to recover DNA is also sometimes caused by DNase contamination of the freshly prepared plasmid DNA, or of the RNase. If miniprep DNA is contaminated with DNase, it can usually be cleaned up by an additional precipitation with ammonium acetate (*UNIT 2.1*) or by phenol extraction (*UNIT 2.1*). This method should not be used with endonuclease A–containing strains (such as HB101).

Lithium miniprep. This procedure eliminates many of the lengthy fractionation steps commonly employed in other minipreps of plasmid DNA and is quite effective for isolating plasmids ≤10 kbp. The high quality of the plasmid DNA obtained by this method has been verified by the fact that no problems are encountered with regard to inhibition of some sensitive restriction enzyme activities—for instance *Nde*I, which is sensitive to trace quantities of impurities (see Fig. 1.6.1). Any DNA cleavage inhibition is likely to be due to contamination of the DNA with cellular debris from the phenol/chloroform interface. However, this can be readily overcome by reducing the volume of DNA in the reaction mixture. The plasmid DNA is suitable for direct transformation of bacteria. If speed is required, DNA recovered from the aqueous phase following phenol/chloroform extraction (after step 4) can be used for transformation. However, such DNA should be used immediately to minimize the likelihood of phenol-mediated modification of DNA.

This technique has been used to purify shuttle vectors from yeast for high-frequency back-transformation in *E. coli* (Ward, 1990).

Anticipated Results

Two to five micrograms of DNA are obtained from 1.5 ml of a culture of cells containing a pBR322-derived plasmid by using either the alkaline lysis miniprep, the boiling protocol, or the lithium method. Three- to five-fold higher yields can be expected from pUC-derived plasmids. DNA yield from alkaline lysis in 96-well microtiter dishes (0.3 ml of culture/well) is ~2 µg for high-copy-number plasmids (e.g., pUC plasmids). All three methods provide plasmid DNA of comparable quality, suitable for use in many applications.

Time Considerations

Using the alkaline lysis procedure, it is possible, with practice, to produce twelve samples

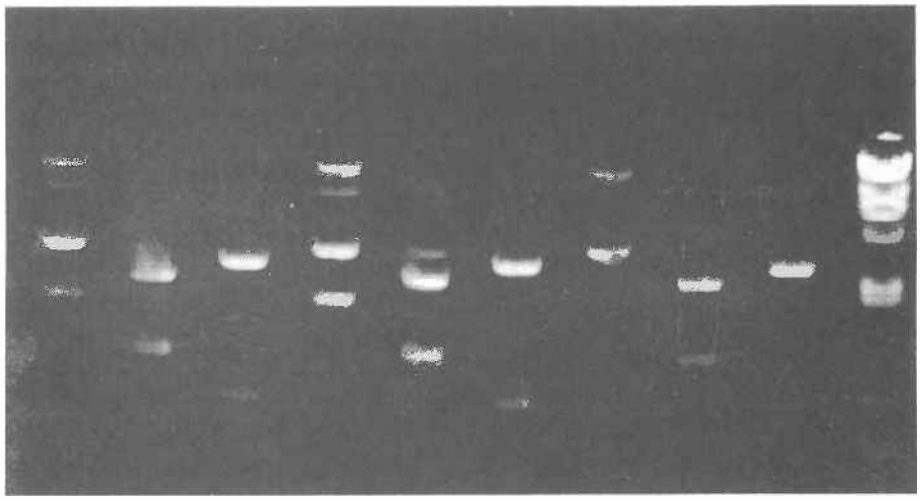


Figure 1.6.1 Restriction mapping of three preparations of plasmid DNA (pVS-WH) from transformant of *E. coli* TB strain. The three clones are numbered 1, 2, and 3 and were treated as follows: **U**, uncleaved DNA; **H-N**, plasmid DNA doubly cut with *Hin*dIII and *Nde*I; **H-S**, plasmid DNA doubly cut with *Hin*dIII and *Sph*I; λ, *Hin*dIII-digested λ DNA. A volume of 2.5 µl of plasmid DNA (about 1 µg) was used for digestion. The samples were separated on a 0.7% agarose gel containing 80 mM Tris-phosphate (pH 8.0) and 1 mM EDTA using Pharmacia Submarine electrophoresis tank with a current of 70 mA for 1 hr. All of the three clones are positive.

of DNA from saturated bacterial cultures in <1 hr. Although it is possible to do a large number of samples in a single day, we recommend using the alkaline lysis in 96-well microtiter dishes for such mass screenings. With the latter protocol, it is easy to process two plates in 4 hr. However, an incubation period of 48 hr is often required for cells to grow to saturation in the wells of the dish.

Starting with saturated cultures, the boiling method allows for the isolation of twelve DNA samples in 1 hr.

The lithium method is extremely quick—DNA samples can be processed in 20 min. With proper organization and availability of the materials, twelve plasmid preparations can be accomplished in substantially less than 1 hr. The entire operation involving plasmid preparation, restriction digestion, and agarose gel electrophoresis can be easily completed within half a day.

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Large-Scale Preparation of Plasmid DNA

Although the need for large quantities of plasmid DNA has diminished as techniques for manipulating small quantities of DNA have improved, occasionally large amounts of high-quality plasmid DNA are desired. This unit describes the preparation of milligram quantities of highly purified plasmid DNA. The first part of the unit describes three methods for preparing crude lysates enriched in plasmid DNA from bacterial cells grown in liquid culture. The second part describes three methods for purifying plasmid DNA in such lysates away from contaminating chromosomal DNA and RNA.

Methods for crude lysate preparation—alkaline lysis (first basic protocol), boiling (first alternate protocol), and Triton lysis (second alternate protocol)—separate chromosomal and plasmid DNA by exploiting the structural differences between these molecules. Plasmids are covalently closed and smaller than chromosomal DNA. When the cell lysate is centrifuged to pellet chromosomal DNA and cellular debris, these differences permit plasmid DNA to remain in the supernatant (see Key References for the theory behind each protocol). All three yield a solution greatly enriched for plasmid DNA but that still contains significant amounts of chromosomal DNA and RNA. These contaminants must be removed if certain procedures, including 5′ end labeling with T4 polynucleotide kinase and transfection of higher eukaryotic cells, are to be performed with the DNA. Accordingly, three procedures are described for purifying plasmid DNA from crude lysates. The second basic protocol, CsCl/ethidium bromide density gradient centrifugation, permits separation by the different capacities of covalently closed plasmid DNA and chromosomal DNA to bind the intercalating agent ethidium bromide. Because binding of ethidium bromide lowers the density of DNA and plasmid DNA can bind less ethidium bromide than chromosomal DNA, plasmid DNA forms bands in a region of greater density (lower in the tube) than chromosomal DNA (see Fig. 1.7.1). The third alternate protocol uses polyethylene glycol (PEG) precipitation and takes advantage of the inverse relationship between macromolecular size and concentration of PEG required for precipitation. The fourth alternate protocol describes two chromatographic methods for purifying plasmid DNA: anion-exchange, which exploits the strong negative charge of nucleic acids, and size-exclusion, which takes advantage of the large size of plasmid DNA molecules relative to other molecules present in the crude lysate.

PREPARATION OF CRUDE LYSATE BY ALKALINE LYSIS

BASIC PROTOCOL

Alkaline lysis is probably the most generally useful plasmid preparation procedure. It is fairly rapid, very reliable, and yields reasonably clean crude DNA that can be further purified by any of the purification methods described in this unit. Plasmid-bearing *E. coli* cells are lysed with lysozyme. The lysate is treated with NaOH/SDS solution and potassium acetate and centifuged to separate plasmid DNA from proteins and chromosomal DNA. The supernatant is treated with isopropanol to precipitate plasmid DNA.

Materials

For recipes, see ***Reagents and Solutions*** *in this unit (or cross-referenced unit); for common stock solutions, see* ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

LB medium or enriched medium (e.g., superbroth or terrific broth; *UNIT 1.1*) containing ampicillin or other appropriate selective agent (Table 1.4.1)
Plasmid-bearing *E. coli* strain
Glucose/Tris/EDTA solution (*UNIT 1.6*)
25 mg/ml hen egg white lysozyme in glucose/Tris/EDTA solution (prepare fresh)
0.2 M NaOH/1% (w/v) SDS [prepare fresh from 10 M NaOH and 10% (w/v) SDS stocks]

3 M potassium acetate solution, pH ~5.5 (see recipe)
Isopropanol
70% ethanol

Sorvall GSA, GS-3, or Beckman JA-10 rotor or equivalent
High-speed centrifuge tubes with ≥20-ml capacity (e.g., Oak Ridge centrifuge tubes)
Sorvall SS-34 or Beckman JA-17 rotor or equivalent

Grow and concentrate cells

1. Inoculate 5 ml LB medium or enriched medium containing selective agent (most commonly ampicillin) with a single colony of *E. coli* containing the desired plasmid. Grow at 37°C with vigorous shaking overnight.

2. Inoculate 500 ml LB medium or enriched medium containing selective agent in a 2-liter flask with ~1 ml of overnight culture. Grow at 37°C until culture is saturated ($OD_{600} \cong 4$).

   To increase yields, maximize aeration using a flask with high surface area (whose volume exceeds the culture volume—i.e., is >2 liters) and baffles and shake at >400 rpm. Alternatively, treat cultures of cells growing logarithmically with chloramphenicol to amplify the plasmids (see Introduction to Plasmid Biology, UNIT 1.5). Growing the bacteria in medium that supports higher cell densities also increases the yield. These media include M9, terrific broth, and LB medium containing 0.1% (w/v) glucose (UNIT 1.1). These media can increase plasmid yields 2- to 10-fold; different plasmids respond to the media differently. Most plasmids commonly used today, particularly derivatives of the pUC series (Fig. 1.5.2), grow at a copy number high enough to routinely yield 1 to 5 mg plasmid DNA from a 500-ml culture grown in LB medium.

   An important consideration when using enriched medium is the method to be used for final purification of plasmid DNA. Increased yield poses no problems when using CsCl/ethidium bromide or PEG purification. However, the capacity of some commercially available chromatography columns—e.g., the Qiagen-tip 2500 (Qiagen) and Wizard Maxiprep (Promega)—is easily exceeded. Therefore, the increased yield of plasmid DNA in the crude lysate will not result in increased recovery from the column. The pZ523 column (5 Prime→3 Prime) does not require that plasmid DNA bind to the column and can be used to purify larger amounts of DNA.

3. Collect cells by centrifuging 10 min at 6000 × *g* (~6000 rpm in Sorvall GSA/GS-3 or Beckman JA-10 rotors), 4°C.

   If necessary the pellets can be stored frozen indefinitely at –20° or –70°C.

4. Resuspend pellet from 500-ml culture in 4 ml glucose/Tris/EDTA solution and transfer to high-speed centrifuge tube with ≥20-ml capacity.

Lyse the cells

5. Add 1 ml of 25 mg/ml hen egg white lysozyme in glucose/Tris/EDTA solution. Resuspend the pellet completely in this solution and allow it to stand 10 min at room temperature.

   Neither glucose nor lysozyme is absolutely necessary for the success of the procedure. Glucose serves as a buffer in step 6 when the pH of the solution is greatly increased by addition of NaOH. Glucose provides buffering in the range of pH 12 and, by preventing the pH from rising too drastically in step 6, increases the efficiency of precipitation in step 7 (when the pH is lowered by addition of potassium acetate).

   Lysozyme assists in the destruction of bacterial cell walls and subsequent release of plasmid DNA. Bacterial debris and soluble proteins are precipitated in step 7. One problem that can reduce recovery of plasmid DNA is inefficient separation of plasmid DNA from cellular debris. Lysozyme helps increase yield by reducing the amount of

plasmid DNA trapped in partially degraded cell material and subsequently lost by precipitation at step 7.

The effort and expense required to include glucose and lysozyme in step 5 is negligible. The efficiency gained in streamlining the procedure by omitting them is also negligible. However, the potential for loss of plasmid DNA when these components are not included is measurable and worth avoiding. It should be noted that some commercially available chromatographic systems (e.g., Qiagen) rely on inefficient bacterial lysis to reduce contamination of plasmid DNA with chromosomal DNA. Although omitting lysozyme reduces the recovery of plasmid DNA, when using these products the manufacturer's recommendations should be followed.

When chromatographic methods are used for final purification of plasmid DNA, it is essential to degrade RNA that contaminates the lysate, and will copurify with plasmid DNA. Treating the lysate with RNase A is the most efficient and economical method for degrading RNA. This can be accomplished at any step in the preparation of crude lysate, it is most convenient to do it at step 5, by adding RNase A to the glucose/Tris/EDTA solution to a final concentration of 50 μg/ml.

6. Add 10 ml freshly prepared 0.2 M NaOH/1% SDS and mix by stirring gently with a pipet until solution becomes homogeneous and clears. Let stand 10 min on ice.

   The solution should become very viscous.

7. Add 7.5 ml of 3 M potassium acetate solution and again stir gently with a pipet until viscosity is reduced and a large precipitate forms. Let stand 10 min on ice.

8. Centrifuge 10 min at 20,000 × *g* (13,000 rpm in Sorvall SS-34; 12,500 rpm in Beckman JA-17), 4°C.

   A large, fairly compact pellet will form; this contains most of the chromosomal DNA, SDS-protein complexes, and other cellular debris. Plasmid DNA remains in the translucent supernatant.

   Addition of ~0.5 ml chloroform before the centrifugation can help reduce floating material.

Precipitate plasmid DNA

9. Decant the supernatant into a clean centrifuge tube. Pour it through several layers of cheesecloth if any floating material is visible. Add 0.6 vol isopropanol, mix by inversion, and let stand 5 to 10 min at room temperature.

   If the supernatant is cloudy or contains floating material, repeat centrifugation (step 8) before adding isopropanol.

10. Recover nucleic acids by centrifuging 10 min at 15,000 × *g* (11,500 rpm in SS-34 rotor; 10,500 rpm in JA-17 rotor), room temperature.

11. Wash the pellet with 2 ml of 70% ethanol; centrifuge briefly at 15,000 × *g*, room temperature, to collect pellet. Aspirate ethanol and dry pellet under vacuum.

    The pellet can be stored indefinitely at 4°C.

ALTERNATE PROTOCOL

PREPARATION OF CRUDE LYSATE BY THE BOILING METHOD

The boiling method is extremely simple and fast but typically yields crude DNA containing more contaminating bacterial DNA and proteins than other methods. In this protocol a bacterial cell lysate is boiled to denature chromosomal DNA and protein. These denatured macromolecules are precipitated by centrifugation, whereas plasmid DNA remains in the supernatant. The plasmid DNA is then precipitated with isopropanol.

Additional Materials

*For recipes, see **Reagents and Solutions** in this unit (or cross-referenced unit); for common stock solutions, see **APPENDIX 2**; for suppliers, see **APPENDIX 4**.*

STET solution (*UNIT 1.6*)
10 mg/ml hen egg white lysozyme in 25 mM Tris·Cl, pH 8.0 (prepare fresh)

Boiling and ice-water baths
Sorvall HB-4 rotor (or equivalent) and appropriate centrifuge tube

1. Grow and concentrate cells as described in basic protocol steps 1 to 3.
2. Resuspend pellet from a 500-ml culture in 20 ml STET solution and transfer to a glass tube or flask.
3. Add 2 ml hen egg white lysozyme and mix solution by inverting several times. Heat to near boiling over an open flame, then incubate 1 min in a boiling water bath.

   Be certain that enough room remains between the top of the solution and the top of the tube to permit safe handling and to prevent boiling over. Submerge the tube deep enough into the boiling water to allow the entire solution to heat rapidly.
4. Place tube into an ice-water bath to cool.
5. Pour solution into centrifuge tube and centrifuge 20 min at ≥25,000 × *g*, preferably in a swinging-bucket rotor (e.g., 12,000 rpm in HB-4), room temperature.

   After boiling, the solution will be extremely viscous due to denatured chromosomal DNA. It will tend to behave as a gooey, semisolid mass. Therefore, be careful not to allow the solution to overflow when pouring it into centrifuge tubes.

   Centrifugation in a swinging-bucket rotor permits concentration of chromosomal DNA and denatured proteins at the bottom of the tube in a more compact pellet than is possible in a fixed-angle rotor. However, fixed-angle rotors can be used—e.g, Sorvall SS-34 at 47,000 × g (20,000 rpm), Beckman JA-17 at 40,000 × g (17,000 rpm), Beckman 70Ti at 200,000 × g (44,000 rpm), or SW-41 at 100,000 × g (25,000 rpm).
6. Decant supernatant to a clean centrifuge tube.

   The supernatant can be used without further treatment for purifying plasmid DNA by CsCl/ethidium bromide equilibrium gradient centrifugation (second basic protocol). If the volume is greater than necessary and inconvenient to handle, plasmid DNA can be precipitated with isopropanol. Precipitation is required for purification by PEG precipitation or column chromatography (third or fourth alternate protocol).
7. Add 0.6 vol isopropanol, mix by inversion, and let stand 5 to 10 min at room temperature.
8. Pellet nucleic acids by centrifuging 10 min at 15,000 × *g* (11,500 rpm in SS-34 rotor; 10,500 rpm in JA-17 rotor), room temperature.
9. Wash the pellet with 2 ml of 70% ethanol. Centrifuge briefly to collect pellet. Aspirate ethanol and dry pellet under vacuum.

   The pellet can be stored indefinitely at 4°C.

PREPARATION OF CRUDE LYSATE BY TRITON LYSIS

ALTERNATE PROTOCOL

The method described below is a modification of that described by Clewell and Helinski (1970, 1972) in which Brij-58 and sodium deoxycholate were used. In this protocol plasmid DNA is extracted from a bacterial cell lysate that has been treated with Triton X-100 (TX-100). This is a very gentle procedure and is therefore useful for isolating very large plasmids such as cosmids. Plasmid DNA can be further purified by any of the methods described in the second part of this unit.

Additional Materials

*For recipes, see **Reagents and Solutions** in this unit (or cross-referenced unit); for common stock solutions, see **APPENDIX 2**; for suppliers, see **APPENDIX 4**.*

Sucrose/Tris/EDTA solution (see recipe)
10 mg/ml hen egg white lysozyme in 25 mM Tris·Cl, pH 8.0 (prepare fresh)
0.5 M EDTA
10 mg/ml DNase-free RNase (*UNIT 3.13*)
Triton lysis solution (see recipe)
1:1 (v/v) buffered phenol/chloroform (*UNIT 2.1*)
24:1 (v/v) chloroform/isoamyl alcohol (*UNIT 2.1*)

1. Grow and concentrate cells as described in basic protocol steps 1 to 3.
2. Resuspend pellet from a 500-ml culture in 5 ml sucrose/Tris/EDTA solution. Transfer to appropriate centrifuge tube (tube should be less than ~⅓ full).
3. Add to tube:

   1.5 ml 10 mg/ml hen egg white lysozyme in 25 mM Tris·Cl
   2 ml 0.5 M EDTA
   25 µl 10 mg/ml DNase-free RNase.

   Let stand 15 min on ice.
4. Overlay with 2.5 ml Triton lysis solution. Mix gently but thoroughly by inversion and let stand 20 min at 4°C.

   This solution should not be vortexed or shaken hard because that will shear chromosomal DNA and prevent it from precipitating in the next step. The solution will become extremely viscous as the cells lyse. Streaks of opaque material will be visible and may remain throughout the incubation.
5. Centrifuge 70 min at 40,000 × *g* (18,000 rpm in Sorvall SS-34; 17,000 rpm in Beckman JA-17), 4°C.
6. Decant supernatant carefully to a clean centrifuge tube.

   Avoid contaminating plasmid DNA with the gelatinous pellet. The pellet contains chromosomal DNA and cellular debris. The pellet may detach from the bottom of the tube, so it is helpful to hold a Pasteur pipet against the mouth of the tube while pouring to prevent the pellet from sliding into the clean tube. The integrity of the pellet varies greatly between preparations; occasionally it is necessary to leave behind some of the viscous supernatant to avoid contaminating it with pellet material.

   This supernatant can be used without further treatment for purifyng plasmid DNA by CsCl/ethidium bromide equilibrium gradient centrifugation (second basic protocol). If the volume is greater than necessary and inconvenient to handle, plasmid DNA can be extracted and precipitated with isopropanol. Extraction and precipitation are required for purification by PEG precipitation or column chromatography (third and fourth alternate protocols).

7. Extract the supernatant with 1:1 buffered phenol/chloroform, then with 24:1 chloroform/isoamyl alcohol. Add 0.6 vol isopropanol to the final aqueous phase and let stand 5 to 10 min at room temperature.

   Phenol extraction and isopropanol precipitation are done as described in UNIT 2.1.

8. Pellet nucleic acids by centrifuging 10 min at 15,000 × *g* (11,500 rpm in SS-34 rotor; 10,500 rpm in JA-17 rotor), room temperature.

9. Wash the pellet with 2 ml of 70% ethanol and centrifuge briefly to collect pellet. Aspirate ethanol and dry pellet under vacuum.

   The pellet can be stored indefinitely at 4°C.

BASIC PROTOCOL

PURIFICATION OF PLASMID DNA BY CsCl/ETHIDIUM BROMIDE EQUILIBRIUM CENTRIFUGATION

This purification procedure yields high-quality plasmid DNA free of most contaminants, but requires the use of ethidium bromide (a mutagen) and often requires long ultracentrifuge runs to establish the density gradient. A crude bacterial cell lysate is mixed with cesium chloride (CsCl) and ethidum bromide and centrifuged to equilibrium. Ethidium bromide is removed by passing plasmid DNA over a cation exchange column, and CsCl is removed by ethanol precipitation.

Materials

*For recipes, see **Reagents and Solutions** in this unit (or cross-referenced unit); for common stock solutions, see **APPENDIX 2**; for suppliers, see **APPENDIX 4**.*

Pellet from crude lysate of plasmid-bearing bacterial cell culture (first basic or first or second alternate protocol)
TE buffer, pH 7.5
Cesium chloride
10 mg/ml ethidium bromide
CsCl/TE solution (see recipe)
Dowex AG50W-X8 cation-exchange resin (see recipe)
TE buffer (pH 7.5)/0.2 M NaCl
100% and 70% ethanol

Beckman VTi65 or VTi80 rotor (or equivalent)
5-ml quick-seal ultracentrifuge tubes
3-ml syringes with 20-G needles

Additional reagents and equipment for ethanol precipitation (*UNIT 2.1*)

CAUTION: Ethidium bromide is a mutagen and environmental hazard. It should be handled carefully with gloves and disposed of properly. Methods for disposal vary between different institutions. Consult the institution's environmental safety office for the preferred means of storage and disposal of ethidium bromide–containing waste.

1. Resuspend pellet from a crude lysate preparation in 4 ml TE buffer. Add 4.4 g CsCl, dissolve, and add 0.4 ml of 10 mg/ml ethidium bromide. If using supernatants resulting from boiling or Triton lysis preparations, add 1.1 g CsCl/ml supernatant and 0.1 ml of 10 mg/ml ethidium bromide/ml supernatant.

   Ethidium bromide will form a complex with protein remaining in the solution to form a deep red flocculent precipitate. This can be removed by centrifuging the lysate–CsCl/ethidium bromide solution 5 min at ~2000 × g, room temperature. After this procedure, the protein–ethidium bromide complex will form a disc at the top of the solution. The solution can be pipetted out from beneath the disc or poured carefully, allowing the floating disc to adhere to the side of the tube.

2. Transfer the solution to a 5-ml ultracentrifuge tube. Top up the tube, if necessary, with CsCl/TE solution and seal tube. Band plasmid by centrifuging 3.5 hr at 500,000 × *g* (77,000 rpm in VTi80 rotor) or ≥14 hr at 350,000 × *g* (65,000 rpm in VTi80 rotor; 58,000 rpm in VTi65 rotor), 20°C.

   This centrifugation must be done at a temperature no lower than 15°C. Because of the high concentration of CsCl and the high centrifugal force necessary to establish the gradient, lower temperatures will cause the CsCl at the bottom of the tube (where the density is highest) to precipitate during the run. CsCl precipitate moves the center of mass towards the bottom of the tube. This can unbalance the rotor and cause breakage of the rotor and destruction of the centrifuge at least, and serious personal injury at worst. Also, equilibrium is achieved more quickly by warmer gradients.

   Other rotors can be used, including fixed-angle and swinging-bucket rotors (e.g., centrifuge ≥24 hr at 56,000 rpm in Ti70 rotor). These rotors require longer centrifugation times than vertical rotors, but allow larger volumes or more samples to be included in the run.

3. Carefully remove the tube from the centrifuge. Insert a 20-G needle gently into the top of the tube. Recover the plasmid band (the lower of the two bands) by inserting a 3-ml syringe with a 20-G needle attached into the side of the tube ~1 cm below the plamid band as shown in Figure 1.7.1. Insert the needle with the beveled side up.

   Do not allow the gradient to be mixed by rough handling or turbulence. Be certain not to cover the top of the first needle with gloved finger. The needle serves to provide an inlet for air to displace the volume of solution being withdrawn.

   If chromosomal DNA has been thoroughly removed in previous steps, only the plasmid band may be visible. Large amounts of plasmid DNA will be visible in the gradient in ordinary light. Smaller amounts can be visualized more easily by side illumination with low-intensity shortwave UV light. Prolonged exposure of the DNA–ethidium bromide complex to UV light will cause damage to the DNA and should be avoided. RNA may be detected as a diffuse region of fluorescence at the bottom of the tube.

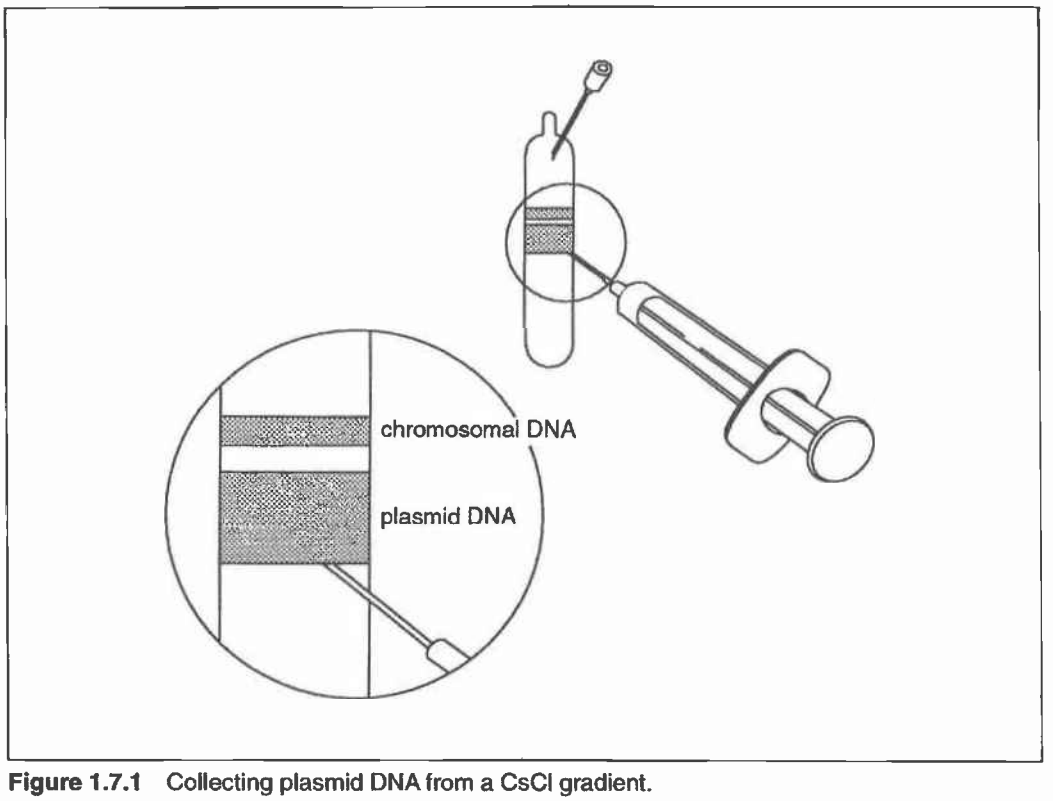

Figure 1.7.1 Collecting plasmid DNA from a CsCl gradient.

CAUTION: *To avoid potentially serious eye injury by UV light, wear UV-blocking glasses or face shield. Wear gloves when handling ethidium bromide.*

Protein–ethidium bromide complexes will pellet on the outside edge of the tube if not removed earlier. To prevent contamination of plasmid DNA, avoid this area when inserting the collection needle.

There should be no resistance in the syringe when drawing off the plasmid DNA. If there is resistance, check that needles are clear. Occasionally the needle will become clogged if a piece of tube enters it. Do not try to draw harder on the syringe, as this may create turbulence in the tube when the obstruction is sucked in and cause mixing of the gradient. Instead, insert another needle and use it to draw off the band. Leave the clogged needle in place in the tube. (If the clogged needle is removed, the tube will empty through the hole that remains.) The air inlet needle can also become clogged; if it does, remove it and allow air to enter through the remaining hole. If plasmid DNA is drawn through a very small opening in a clogged needle, it may be sheared.

4. If higher-purity plasmid DNA is required, perform a second ultracentrifugation to eliminate any contaminating RNA or chromosomal DNA. Add plasmid DNA band to another ultracentrifuge tube, top up with CsCl/TE solution containing 1.0 mg/ml ethidium bromide, and repeat steps 2 and 3.

5. Pour a Dowex AG50W-X8 column, 1.5 to 2 times the volume of the plasmid DNA/ethidium bromide solution, in a glass or plastic column. Pass several volumes of TE buffer/0.2 M NaCl through the column to wash and equilibrate it.

   The column can be set up in a Pasteur pipet plugged with a little glass wool or in a commercially purchased plastic column.

6. Load the plasmid DNA/ethidium bromide solution directly from syringe to top of resin bed without disturbing the resin.

7. Begin collecting the solution flowing through the column immediately after loading the plasmid solution. Wash the column with a volume of TE buffer/0.2 M NaCl equal to twice that of the volume of plasmid solution loaded.

   The final volume collected from the column should be three times the volume of plasmid DNA solution removed from the gradient.

   As the plasmid DNA flows through the column the ethidium bromide will be retained in the resin and form a red band at the top. All of the DNA will flow through in the volume recommended. This procedure will dilute the CsCl sufficiently to allow the DNA to be precipitated.

   Ethidium bromide can also be removed by extracting the plasmid DNA–ethidium bromide solution with an equal volume of TE-saturated n-butanol (UNIT 2.1). Shake the tube or vortex it vigorously to maximize the efficiency of extraction. Remove the organic upper phase and extract the aqueous phase repeatedly until no red color remains. Dilute the solution 3-fold with TE buffer to dilute the CsCl. This procedure generates contaminated organic solvent waste. Follow correct procedure for its disposal.

8. Ethanol precipitate plasmid DNA, using 2 vol of 100% ethanol at room temperature or –20°C, and centrifuge 10 min at 10,000 × *g*, 4°C.

   Do not cool this solution below –20°C, as this may cause the CsCl to precipitate.

   An alternative to ethanol precipitation at this step is to dialyze the plasmid DNA, from which the ethidium bromide has been removed, against 500 to 1000 vol TE buffer. Dialysis buffer should be changed at least twice with ≥2 hr between changes at room temperature, or 4 hr at 4°C (see APPENDIX 3).

9. Wash pellet with 70% ethanol and dry under vacuum. Resuspend pellet in TE buffer and store at 4°C.

PLASMID DNA PURIFICATION BY PEG PRECIPITATION

ALTERNATE PROTOCOL

Polyethylene glycol (PEG) precipitation is a rapid, reliable, and convenient method for plasmid DNA purification. It can be stopped at any step without affecting the ultimate recovery of plasmid DNA. No ultracentrifugation is required and the use of ethidium bromide (a mutagen) is avoided. RNA and chromosomal DNA contaminants are removed from a crude lysate pellet by treating it with RNase, NaOH/SDS, and potassium acetate. Plasmid DNA is extracted from the supernatant and precipitated with PEG. This method is suitable for preparing plasmid DNA for procedures that require plasmid DNA free of any contaminants.

Additional Materials

*For recipes, see **Reagents and Solutions** in this unit (or cross-referenced unit); for common stock solutions, see **APPENDIX 2**; for suppliers, see **APPENDIX 4**.*

Glucose/Tris/EDTA solution (*UNIT 1.6*)
0.2 M NaOH/1% (w/v) SDS [prepare fresh from 10 M NaOH and 10% (w/v) SDS stocks]
10 mg/ml DNase-free RNase (*UNIT 3.13*)
3 M potassium acetate solution, pH ~5.5 (see recipe)
Buffered phenol (*UNIT 2.1*)
24:1 (v/v) chloroform/isoamyl alcohol (*UNIT 2.1*)
10 M ammonium acetate
100% and 70% ethanol
TE buffer, pH 7.5
PEG solution (see recipe)
3 M sodium acetate, pH 5.5

Sorvall SS-34 or Beckman JA-17 rotor (or equivalent)
Sorvall HB-4 rotor

Remove contaminants from crude lysate pellet

1. Resuspend the pellet obtained in the final step of crude lysate preparation in 1 ml glucose/Tris/EDTA solution.
2. Add RNase to a final concentration of 20 µg/ml and incubate 20 min at 37°C.
3. Add 2 ml freshly prepared 0.2 M NaOH/1% SDS, mix by inverting the tube, and let stand 5 to 10 min at room temperature.
4. Add 1.5 ml of 3 M potassium acetate solution, mix by inverting the tube, and let stand 5 to 10 min at room temperature.
5. Centrifuge 10 min at 20,000 × *g* (11,000 rpm in SS-34 rotor; 12,500 rpm in HB-4 or JA-17 rotor), room temperature.

   If restriction fragments of the plasmid are to be prepared for use as probes in filter hybridizations, gel purification will rid the desired fragments of any contaminating traces of chromosomal DNA. In that case steps 1 to 5 can be omitted. Resuspend the pellet from the crude lysate in 4 ml TE buffer and add RNase to 20 µg/ml final. Incubate 20 min at 37°C and treat as described in steps 7 to 12 of this protocol.

6. Transfer the supernatant to a clean tube.

   The white precipitate is primarily SDS-potassium complex. It includes any chromosomal DNA that remained in the crude lysate. The amount remaining after crude lysate preparation varies. It may not be necessary to remove the chromosomal DNA completely, depending on the procedures to which the plasmid DNA is to be subjected. However, some procedures such as hybrid selection (UNIT 6.8), require that plasmid DNA be free of any contaminants.

7. Extract plasmid DNA with buffered phenol, then with 24:1 chloroform/isoamyl alcohol.
8. Add ¼ vol 10 M ammonium acetate (2 M final concentration) to the aqueous phase and mix. Add 2 vol 100% ethanol and place tube in dry ice for 10 min.
9. Recover plasmid DNA by centrifuging 10 min at 10,000 × *g* (8000 rpm in HB-4 or SS-34 rotor; 8500 rpm in JA-17 rotor), 4°C.
10. Wash the pellet with 70% ethanol and dry briefly under vacuum.

PEG precipitate plasmid DNA

11. Resuspend the pellet in 2 ml TE buffer and add 0.8 ml PEG solution. Incubate 1 to 15 hr at 0°C.

    The percentage of plasmid DNA recovered with PEG precipitation increases with time of incubation at 0° to 4°C. Usually ≥50% (~0.5 to 3 mg) can be recovered by centrifuging the PEG-precipitated DNA solution after 1 hr at 0°C. Incubating the supernatant ≥12 hr at 4°C will permit complete recovery of remaining plasmid DNA while plasmid DNA collected after the first centrifugation can be used for other procedures.

12. Recover plasmid DNA by centrifuging 20 min at 10,000 × *g* (8000 rpm in HB-4 or SS-34 rotor; 8500 rpm in JA-17 rotor; 10,000 rpm in most microcentrifuges), 4°C.
13. Resuspend pelleted plasmid DNA in 1 ml TE buffer. Ethanol precipitate plasmid DNA using 3 M sodium acetate, pH 5.5.

ALTERNATE PROTOCOL

PLASMID DNA PURIFICATION BY ANION-EXCHANGE OR SIZE-EXCLUSION CHROMATOGRAPHY

Chromatographic methods for purifying plasmid DNA take advantage of distinctions between the physical properties of plasmid DNA and those of molecules that copurify with it in the crude lysate. Nucleic acids are negatively charged and can therefore be purified away from contaminants using anion-exchange chromatography (see *UNIT 2.14* for a protocol and discussion of one anion-exchange method). Similarly, the large size of plasmid DNA allows it to be purified away from smaller contaminants by gel-filtration chromatography. The specific properties of most matrices provided by commercial suppliers are unknown, due to the reluctance of the suppliers to divulge proprietary information, but they typically exploit one or both of these methods.

This protocol describes modifications for preparing a crude lysate for chromatographic purification of plasmid DNA and discusses features of three commercially available columns. Because no single protocol is appropriate for all of the chromatography methods, it is important to adhere to the manufacturer's suggested methodology. Most columns are supplied as kits that include reagents for preparing crude lysate using alkaline lysis (first basic protocol); some manufacturers will provide the columns separately.

Preparation of crude lysate. It is unnecessary, and may be futile, to attempt to maximize cell density and plasmid DNA concentration as described in first basic protocol step 2 if the capacity of the column to be used will be exceeded. The pZ523 column (5 Prime→3 Prime) has a capacity of 4 to 5 mg plasmid DNA; the Qiagen-tip 2500 (Qiagen) and Wizard Maxiprep (Promega) columns have capacities of ~1 to 2 mg.

Successful chromatographic purification of DNA using the Qiagen-tip 2500 and Wizard Maxiprep columns requires that the bacterial cells be incompletely lysed, so lysozyme should be omitted from the preparation of crude lysate.

The most frequent contaminant of plasmid DNA prepared by chromatographic methods is high-molecular-weight RNA. This contamination is reduced by adding 50 µg/ml RNase A (from frozen 1 mg/ml stock, *UNIT 3.13*) to the resuspended cell pellet. The reagents provided with Qiagen-tip 2500 and Wizard Maxiprep columns contain RNase A. However, RNase A is stable for no more than several months at 4°C, the storage temperature of the working solutions. If the reagents are used for >4 to 6 months, add fresh RNase A to give a total concentration of 100 µg/ml. The reagents provided by 5 Prime→3 Prime include a mixture of RNase A and RNase T1. The manufacturer suggests adding the RNases to a resuspended crude lysate pellet (first basic protocol, step 11) because the pellet is resuspended in a smaller volume at this step and less RNase is required.

Column flow is greatly impeded or completely prevented by the presence of solid material in the lysate when it is loaded onto the column. The most common source of solids is floating material from the precipitation (first basic protocol, step 7). To be certain that no floating material is loaded onto the column, decant the supernatant through cheesecloth, add chloroform before centrifugation, or recentrifuge the supernatant.The Qiagen protocol allows the lysate to be loaded on the column directly following removal of the protein and cellular debris by precipitation and centrifugation (first basic protocol, steps 7 to 9). The Wizard Maxiprep and pZ523 protocols require the crude lysate plasmid DNA to be precipitated with isopropanol. This reduces the volume of material to be loaded on the column, and allows the buffer in which plasmid DNA is loaded to be optimized for the column. Isopropanol precipitation can be used with either of the alternate protocols for preparation of crude lysate, and the final pellet can be resuspended in the buffer appropriate for the chromatographic matrix to be used.

Column capacity. Plasmid DNA binding capacity is the limiting factor in the use of most popular columns. Overloaded columns will not result in increased yields of plasmid DNA. The pZ523 column has a capacity of 4 to 5 mg and does not require that DNA bind to the column, so overloading is less likely to be a problem. The standard protocols for most other commercial columns are adjusted to provide a "good" yield for a plasmid that is maintained at a moderate copy number when cells that bear it are grown in ~500 ml LB medium. To optimize recovery, bacterial culture volume, plasmid copy number, and the culture medium must be adjusted to the capacity of the column matrix. Larger culture volumes are appropriate when preparing DNA from cosmids or low-copy-number plasmids (e.g., pBR322 and derivatives). When preparing DNA from high-copy-number plasmids such as pUC and its derivatives; culture volumes ¼ to ½ the standard volume may be appropriate. When using media that support growth to high cell density such as Terrific Broth (*UNIT 1.1*; Tartoff and Hobbs, 1987), culture volumes ¼ to 1/10 the standard may be appropriate. The protocol provided by Qiagen includes a table of recommended culture volumes.

Plasmid DNA exceeding the capacity of the column will in no way prevent recovery of DNA. The excess DNA will simply run through the column and be discarded. One way to increase the yield is to recover and save the material that flows through the column when it is initially loaded. Some columns can be regenerated following elution of the plasmid DNA and the initial flowthrough reloaded. This is possible with the Qiagen column, as suggested in the protocol provided by the manufacturer; repeating chromatography of the initial flowthrough material can increase yield nearly 2-fold in some instances. To avoid contamination with different plasmids, use a regenerated column only to purify the same plasmid for which it was originally used. The Qiagen column can be reused because it is run by gravity flow and the matrix is not compacted. Columns such as Wizard Maxiprep and pZ523, which require vacuum and centrifugation respectively, collapse during use and cannot be reused. In this case, excess DNA in the flowthrough can be purified on a second column of the same type.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

CsCl/TE solution

100 ml TE buffer, pH 7.5
100 g CsCl
Store indefinitely at room temperature

Dowex AG50W-X8 cation exchange resin

Prepare large batches (200 to 400 ml packed resin) of Dowex AG50W-X8 resin (100- to 200-mesh; Bio-Rad) by performing the following series of washing steps. Use a large Buchner funnel and filter paper to collect the resin between changes of wash solution.

1. Wash resin in ≥10 vol of 0.5 N NaOH until no color is observed in wash solution (resin will retain its buff color).
2. Wash with 5 to 10 vol of 0.5 N HCl.
3. Wash with 5 to 10 vol of 0.5 M NaCl.
4. Wash with 5 to 10 vol of distilled H_2O.
5. Wash with 5 to 10 vol of 0.5 N NaOH.
6. Wash with distilled H_2O until pH = 9.
7. Store prepared resin indefinitely in 0.5 M NaCl/0.1 M Tris, (pH 7.5), at 4°C.

Polyethylene glycol (PEG) solution

30% (w/v) PEG 8000
1.6 M NaCl
Store indefinitely at 4°C

Potassium acetate solution (3 M), pH ~5.5

294 g potassium acetate (3 M final)
50 ml 90% formic acid (1.18 M final)
H_2O to 1 liter
Store indefinitely at room temperature

Sucrose/Tris/EDTA solution

25% (w/v) sucrose
50 mM Tris·Cl, pH 8.0
100 mM EDTA, pH 8.0
Store indefinitely at 4°C

Triton lysis solution

3% (v/v) Triton X-100
200 mM EDTA, pH 8.0
150 mM Tris·Cl, pH 8.0
Store indefinitely at 4°C

COMMENTARY

Critical Parameters and Troubleshooting

Preparation of crude lysate by alkaline lysis. This is a reliable procedure and one in which few irretrievable disasters can occur. One potential problem is failure of chromosomal DNA and proteins to precipitate after addition of potassium acetate solution. The cause of this is probably improper pH of the potassium acetate solution. The NaOH/SDS solution denatures linear (chromosomal) DNA. When the solution is neutralized in the presence of a high salt concentration (as when potassium acetate solution is added), the linear DNA precipitates. This precipitation presumably is due to interstrand reassociation of denatured, linear DNA molecules at multiple sites. It results in the formation of a large, insoluble DNA network. Protein–SDS complexes also precipitate under these conditions. If the pH of the potassium acetate solution is not ~5.5, these precipitates will not form. If precipitation fails to occur because the pH is incorrect, the preparation can be saved by adding concentrated formic or acetic acid dropwise to the solution. Mix after each addition until the viscosity decreases, which will happen suddenly. A precipitate will then appear.

Preparation of crude lysate by the boiling method. The drawback to this procedure is that if it fails there are few chances for recovery. The only easily assayed step is the final recovery of plasmid DNA. Failure is often caused by inactive lysozyme or incorrect boiling time. An indication of failure will be that the solution is not extremely viscous following step 4. The remedy for inactive lysozyme is to try a new preparation of lysozyme. The optimal boiling time may vary slightly between bacterial strains. Using a strain for which the correct time is already known is the simplest remedy to this problem. Alternatively, the correct time may be determined empirically by performing the boiling miniprep procedure in *UNIT 1.6*.

Preparation of crude lysate by Triton lysis. This method is much gentler than the others described in this unit because of the relatively mild conditions used to disrupt the cells (i.e., use of Triton X-100 and lysozyme rather than boiling or severe changes in ionic strength and pH). Because the conditions used are near the lower limits of their effectiveness, solutions must be prepared correctly and the lysozyme must be active. Although powdered lysozyme is stable for long periods when stored properly, it is occasionally necessary to purchase and use a new bottle. The solution should become extremely viscous when Triton lysis solution is added, indicating that lysis has occurred. Centrifugation is also critical for separating plasmid DNA from the bulk of contaminants. If the pellet is not reasonably firm at this stage it will pour out of the tube when the plasmid DNA–containing supernatant is decanted. If this happens, repeat the centrifugation. If it happens routinely, increase the reagent volumes proportionately throughout the procedure or centrifuge for longer times or at higher *g* forces, if necessary using an ultracentrifuge.

Plasmid DNA purification by CsCl/ethidium bromide centrifugation. It is important that the density of the DNA–CsCl/ethidium bromide solution be correct for this procedure to work. Therefore, be precise (using graduated plastic tubes) when measuring the solution volume as this determines the amount of CsCl to be added. If the amount of added CsCl is incorrect, the position of the bands will be high (if too much was added) or low (if too little was added). Ideally the bands should appear slightly above the center of the tube. It is also important to allow sufficient time for the establishment of the gradient during centrifugation. If the bands appear diffuse at the end of the run, resolution is not adequate and centrifugation should be continued, In a gradient that has achieved equilibrium, bands are well defined.

Occasionally an ultracentrifuge tube breaks and its contents leak into the rotor compartment. This can occur if the tube is defective or if it is not filled or sealed properly. If most of the solution remains in the rotor compartment it can be pipetted into a clean tube, topped up with CsCl/TE solution, and centrifuged again. The quality of the DNA should not be affected, although the yield will be lower due to loss of material. The rotor should be rinsed with warm water if it comes in contact with CsCl solution. Cesium chloride is very corrosive and can cause pitting and weakening of the rotor. The centrifuge chamber should be inspected after every run, and cleaned with warm water and dried thoroughly if red stains are evident.

If the CsCl precipitates, as evidenced by a large, white, crystalline pellet observed when precipitating plasmid DNA in the final steps, warm the solution to room temperature and centrifuge it at room temperature. If this problem persists, be certain the solution is diluted 3-fold with TE buffer before adding ethanol.

Plasmid DNA purification by PEG precipitation. The purity of plasmid DNA obtained by polyethylene glycol (PEG) precipitation of crude bacterial lysate depends on the amount of chromosomal DNA remaining in the solution when the PEG solution is added. Steps 1 to 5 of the third alternate protocol remove any remaining traces of chromosomal DNA. These steps are not necessary for PEG precipitation to succeed. For size fractionation by PEG to be effective, the concentration of DNA in the crude lysate must be >10 μg/ml. This is not a concern when applied to plasmid DNA purification, where the concentrations should be orders of magnitude greater than that figure.

Plasmid DNA purification by anion-exchange or size-exclusion chromatography. The columns available in kit form from a number of manufacturers are quite reliable. Almost all necessary reagents are provided, including common buffers such as TE buffer. Because the composition of the matrix is undisclosed, it is impossible to evaluate the procedure carefully and attempt to optimize it. Therefore, users of kits are strongly encouraged to follow the manufacturer's recommended procedures. Additional discussion of one type of anion-exchange procedure (Qiagen) can be found in the Commentary of *UNIT 2.14*.

A major drawback to some prepared columns is the limited capacity of the matrix. A 500-ml culture of plasmid-containing bacteria can often yield 2 to 8 mg of plasmid DNA in the crude lysate. Some columns routinely yield only 500 to 1000 μg purified plasmid DNA, which is adequate for most purposes. Recovering the column flowthrough and rechromatographing it is the most practical method of increasing recovery of plasmid DNA. Not all columns can be reused, however, and recovery may require use of additional columns. Alternatively, smaller culture volumes can be used as suggested. If optimal recovery of DNA is desired, CsCl/ethidium bromide centrifugation or PEG precipitation should be used.

DNA obtained from chromatographic purification of plasmid DNA is comparable in quality to that prepared by the other methods. It is of sufficient purity for virtually any procedure for which it can be used. The two most frequent contaminants are chromosomal DNA and high-molecular-weight RNA. These contaminants may be detected by the presence of large, diffuse ethidium bromide–binding material in agarose gel electrophoresis of purified plasmid DNA. To prevent such contamination, follow the manufacturer's suggestions for the use of lysozyme and RNase.

A vacuum manifold is required for the Wizard Maxiprep column, and the pZ523 column must be centrifuged in a swinging-bucket rotor at 1100×*g*. Otherwise only common laboratory equipment is required for chromatographic purification of plasmid DNA. Another consideration with commercial kits is the large amount of packaging material and waste. In addition to the plastic columns and excess packaging, kits contain standard reagents supplied in plastic bottles. These reagents are solutions of buffers, salts, ethanol, and detergent—all of which can be, and usually are, prepared in the lab. Some suppliers will provide the column without the reagents.

The toxicity of DNA prepared by several methods has been assessed by performing a biological assay. Crude lysate from a 1-liter culture of plasmid-containing bacteria was prepared by the alkaline lysis procedure and divided into four equal aliquots. The aliquots were then subjected to purification by CsCl/ethidium bromide centifugation, PEG precipitation, or chromatography on Qiagen and pZ523 columns. Purified plasmid DNA was injected into *Drosophila* embryos, and the frequency of germline transformation and killing of injected embryos was determined. No significant differences attributable to the method of purification were observed.

Anticipated Results

Most plasmids currently used are derivatives of the pUC series (Fig. 1.5.2). These plasmids contain an origin of replication significantly more efficient than that of the previous generation of pBR322-derived plasmids. This allows recovery of 1 to 5 mg of plasmid DNA (free of contaminating bacterial products) from a 500-ml culture following any of the crude lysate preparation methods or PEG precipitation. Purification by CsCl/ethidium bromide density gradient centrifugation yields 75% to 90% the amount of plasmid DNA obtained using PEG precipitation. Yields obtained from column chromatography are limited by the capacity of the column and are generally <1 mg.

Time Considerations

Cell growth and concentration require one overnight growth period to collect the starting 5-ml culture and most of the next day for its outgrowth and concentration. Crude lysate

preparation can be completed in 90 min using alkaline lysis, ~35 min using the boiling method, and 2.5 to 3 hr using Triton lysis. Plasmid DNA purification by CsCl/ethidium bromide density gradient centrifugation takes 4 hr to 3 days depending upon the quality of plasmid DNA desired and the type of centrifuge and rotor used. PEG precipitation of the crude lysate can yield pure plasmid DNA in as little as 2 hr; however, complete recovery may require 13 to 16 hr. Column chromatography takes 30 to 90 minutes. High-quality plasmid DNA recovery from an *E. coli* strain containing the desired plasmid using any combination of these procedures takes 1 day to 1 week.

In addition to the total time necessary to obtain pure plasmid DNA, it is important to consider the hands-on time required for individual steps of the different procedures. PEG precipitation can yield pure plasmid DNA 4 or 5 hr after harvesting cells but requires direct attention and manipulations every 10 to 30 min. Purification using CsCl/ethidium bromide centrifugation takes 6 to 18 hr after harvesting cells, but the final 4-5 hr are taken up by a centrifugation step that requires no direct attention. Column chromotography can yield purified DNA within 3 hr of harvesting cells but requires constant attention. Therefore, if pure plasmid DNA is needed the same day the large culture is harvested, it is perhaps best to prepare crude lysates by alkaline lysis and purify plasmid DNA by PEG precipitation, collecting the PEG precipitate after ~1 hr at 0°C, or by chromatography. If it has been a long day and the priority is to go home, crude lysate can be prepared by the boiling method and plasmid DNA purified by the CsCl/ethidium bromide centrifugation. The variety of methods and the opportunity to interrupt them at different steps (such as at the precipitation steps) facilitates selection of methods most convenient for specific work situations.

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Introduction of Plasmid DNA into Cells

Transformation of *E. coli* can be achieved using any of the four protocols in this unit. The first method (see Basic Protocol 1) using calcium chloride gives good transformation efficiencies, is simple to complete, requires no special equipment, and allows storage of competent cells. The one-step method (see Alternate Protocol 1) is considerably faster and also gives good transformation efficiencies (although they are somewhat lower). However, because it was developed relatively recently, its reproducibility and reliability are not as well established.

If considerably higher transformation efficiencies are needed, the third method (see Basic Protocol 2) using electroporation should be followed. Although this procedure is simple, fast, and reliable, it requires an electroporation apparatus. As in the calcium chloride protocol, prepared cells can be stored. The final method described (see Alternate Protocol 2) describes an adaptation based on electroporation that allows direct transfer of vector DNA from yeast into *E. coli*.

TRANSFORMATION USING CALCIUM CHLORIDE

BASIC PROTOCOL 1

Escherichia coli cells are grown to log phase. Cells are concentrated by centrifugation and resuspended in a solution containing calcium chloride. Exposure to calcium ions renders the cells able to take up DNA, or *competent*. Plasmid DNA is mixed with the cells and presumably adheres to them. The mixture of DNA and cells is then heat shocked, which allows the DNA to efficiently enter the cells. The cells are grown in nonselective medium to allow synthesis of plasmid-encoded antibiotic resistance proteins, then plated on antibiotic-containing medium to allow identification of plasmid-containing colonies.

Materials

Single colony of *E. coli* cells
LB medium (*UNIT 1.1*)
$CaCl_2$ solution (see recipe), ice cold
LB plates (*UNIT 1.1*) containing ampicillin (Table 1.4.1)
Plasmid DNA (*UNITS 1.6 & 1.7*)

Chilled 50-ml polypropylene tubes
Beckman JS-5.2 rotor or equivalent
42°C water bath

Additional reagents and equipment for growth of bacteria in liquid media (*UNIT 1.2*)

NOTE: All materials and reagents coming into contact with bacteria must be sterile.

Prepare competent cells

1. Inoculate a single colony of *E. coli* cells into 50 ml LB medium. Grow overnight at 37°C with moderate shaking (250 rpm; see *UNIT 1.2*).

   Alternatively, grow a 5-ml culture overnight in a test tube on a roller drum.

2. Inoculate 4 ml of the culture into 400 ml LB medium in a sterile 2-liter flask. Grow at 37°C, shaking at 250 rpm, to an OD_{590} of 0.375.

   This procedure requires that cells be growing rapidly (early- or mid-log phase). Accordingly, it is very important that the growing cells have sufficient air. A 1-liter baffle flask can be used instead of the 2-liter flask. Overgrowth of culture (beyond OD_{590} of 0.4) decreases the efficiency of transformation.

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3. Aliquot culture into eight 50-ml prechilled, sterile polypropylene tubes and leave the tubes on ice 5 to 10 min.

   Cells should be kept cold for all subsequent steps.

   Larger tubes or bottles can be used to centrifuge cells if volumes of all subsequent solutions are increased in direct proportion.

4. Centrifuge cells 7 min at 1600 × *g* (3000 rpm in JS-5.2), 4°C. Allow centrifuge to decelerate without brake.

   We have not attempted to determine whether deceleration without braking is critical to this procedure. However, we do not use the brake for this step or for the subsequent centrifugation steps.

5. Pour off supernatant and resuspend each pellet in 10 ml ice-cold $CaCl_2$ solution.

   Resuspension should be performed very gently and all cells kept on ice.

6. Centrifuge cells 5 min at 1100 × *g* (2500 rpm), 4°C. Discard supernatant and resuspend each pellet in 10 ml ice-cold $CaCl_2$ solution. Keep resuspended cells on ice for 30 min.

7. Centrifuge cells 5 min at 1100 × *g*, 4°C. Discard supernatant and resuspend each pellet in 2 ml ice-cold $CaCl_2$ solution.

   It is important to resuspend this final pellet well. The suspension may be left on ice for several days. For many strains (e.g., DH1) competency increases with increasing time on ice, and reaches a maximum at 12 to 24 hr. This is not true for MC1061 cells, which should be frozen immediately.

8. Dispense cells into prechilled, sterile polypropylene tubes (250-µl aliquots are convenient). Freeze immediately at –70°C.

Assess competency of cells

9. Use 10 ng of pBR322 to transform 100 µl of competent cells according to the steps provided below. Plate appropriate aliquots (1, 10, and 25 µl) of the transformation culture on LB/ampicillin plates and incubate at 37°C overnight.

10. Calculate the number of transformant colonies per aliquot volume (µl) × 10^5: this is equal to the number of transformants per microgram of DNA.

    Transformation efficiencies of 10^7 to 10^8 and 10^6 to 10^7 are obtained for E. coli MC1061 and DH1, respectively. Competency of strains decreases very slowly over months of storage time.

Transform competent cells

11. Aliquot 10 ng of DNA in a volume of 10 to 25 µl into a sterile 15-ml round-bottom test tube and place on ice.

    Plasmid DNA can be used directly from ligation reactions. When this is done, more DNA is usually used. However, if there is >1 µg of DNA in the ligation reaction, or if the ligation reaction is from low gelling/melting temperature agarose, it is wise to dilute the ligation mix (see UNIT 3.16).

12. Rapidly thaw competent cells by warming between hands and dispense 100 µl immediately into test tubes containing DNA. Gently swirl tubes to mix, then place on ice for 10 min.

    Competent cells should be used immediately after thawing. Remaining cells should be discarded rather than refrozen.

13. Heat shock cells by placing tubes into a 42°C water bath for 2 min.

    Alternatively, incubate at 37°C for 5 min.

14. Add 1 ml LB medium to each tube. Place each tube on a roller drum at 250 rpm for 1 hr at 37°C.

15. Plate aliquots of transformation culture on LB/ampicillin or other appropriate antibiotic-containing plates.

    It is convenient to plate several different dilutions of each transformation culture. The remainder of the mixture can be stored at 4°C for subsequent platings.

16. When plates are dry, incubate 12 to 16 hr at 37°C.

ONE-STEP PREPARATION AND TRANSFORMATION OF COMPETENT CELLS

ALTERNATE PROTOCOL 1

This procedure is considerably easier than Basic Protocol 1 because it eliminates the need for centrifugation, washing, heat shock, and long incubation periods (Chung et al., 1989). Moreover, competent cells made by this simple procedure can be directly frozen at –70°C for long-term storage. A variety of strains can be made competent by this procedure, and the transformation frequency can be as high as that achieved by Basic Protocol 1. However, frequency is considerably lower than can be obtained by electroporation.

Additional Materials *(also see Basic Protocol 1)*

2× transformation and storage solution (TSS; see recipe), ice cold
LB medium (*UNIT 1.1*) containing 20 mM glucose

1. Dilute a fresh overnight culture of bacteria 1:100 into LB medium and incubate at 37°C until the cells reach an OD_{600} of 0.3 to 0.4.

   The procedure will work if cells are harvested at other stages of the growth cycle (including stationary phase), but with reduced efficiency.

2. Add a volume of ice-cold 2× TSS equal to that of the cell suspension, and gently mix on ice.

   For long-term storage, freeze small aliquots of the suspension in a dry ice/ethanol bath and store at –70°C. To use frozen cells for transformation, thaw slowly and then use immediately.

   Cells can also be used if pelleted by centrifugation 10 min at 1000 × g, 4°C, and this may increase the frequency of transformation (according to Chung et al., 1989). Discard supernatant and resuspend cell pellet at one-tenth of original volume in 1× TSS (prepared by diluting 2× TSS). Proceed with transformation as in step 3.

3. Add 100 µl competent cells and 1 to 5 µl DNA (0.1 to 100 ng) to an ice-cold polypropylene or glass tube. Incubate 5 to 60 min at 4°C.

   As is the case for related procedures, the transformation frequency as measured by transformants/µg DNA is relatively constant at amounts of DNA <10 ng. However, the frequency decreases at higher concentrations. The time of incubation at 4°C is relatively unimportant.

4. Add 0.9 ml LB medium containing 20 mM glucose and incubate 30 to 60 min at 37°C with mild shaking to allow expression of the antibiotic resistance gene. Select transformants on appropriate plates.

   It is unnecessary to heat shock the transformation mixture. The expected transformation frequency should range between 10^6 and 10^7 colonies/µg DNA.

BASIC PROTOCOL 2

HIGH-EFFICIENCY TRANSFORMATION BY ELECTROPORATION

Electroporation with high voltage is currently the most efficient method for transforming *E. coli* with plasmid DNA. The procedure described may be used to transform freshly prepared cells or to transform cells that have been previously grown and frozen. With freshly grown cells, it routinely gives more than 10^9 bacterial transformants per microgram of input plasmid DNA.

Materials

Single colony of *E. coli* cells
LB medium (*UNIT 1.1*)
H_2O, ice cold
10% glycerol, ice cold
SOC medium (see recipe)
LB plates (*UNIT 1.1*) containing antibiotics (Table 1.4.1)

1-liter centrifuge bottle, 50-ml narrow-bottom polypropylene tube, and microcentrifuge tubes, chilled ice cold
Beckman J-6M centrifuge (or equivalent)
Beckman JS-4.2 rotor (or equivalent) and adaptors for 50-ml narrow-bottom tubes
Electroporation apparatus with a pulse controller or 200- or 400-ohm resistor
Chilled electroporation cuvettes, 0.2-cm electrode gap

Additional reagents and equipment for growth of bacteria in liquid media (*UNIT 1.2*)

NOTE: All materials and reagents coming into contact with bacteria must be sterile.

Prepare the cells

1. Inoculate a single colony of *E. coli* cells into 5 ml LB medium. Grow 5 hr to overnight at 37°C with moderate shaking (see *UNIT 1.2*).
2. Inoculate 2.5 ml of the culture into 500 ml LB medium in a sterile 2-liter flask. Grow at 37°C, shaking at 300 rpm, to an OD_{600} of ~0.5 to 0.7.

   Best results are obtained by harvesting cells at an OD_{600} of ~0.5 to 0.6.
3. Chill cells in an ice-water bath 10 to 15 min and transfer to a prechilled 1-liter centrifuge bottle.

   Cells should be kept at 2°C for all subsequent steps.
4. Centrifuge cells 20 min at 4200 rpm in Beckman J-6M, 2°C.
5. Pour off supernatant and resuspend the pellet in 5 ml ice-cold water. Add 500 ml ice-cold water and mix well. Centrifuge cells as in step 4.
6. Pour off supernatant immediately and resuspend the pellet by swirling in remaining liquid.

   Because the pellet is very loose, the supernatant must be poured off immediately. The pellet can be made tighter by substituting ice-cold sterile HEPES (1 mM, pH 7.0) for the ice-cold water in step 5.
7. Add another 500 ml ice-cold water, mix well, and centrifuge again as in step 4.
8. Pour off supernatant immediately and resuspend the pellet by swirling in remaining liquid.

9a. If fresh cells are to be used for electroporation, place suspension in a prechilled, narrow-bottom, 50-ml polypropylene tube, and centrifuge 10 min at 4200 rpm in Beckman J-6M centrifuge with JS-4.2 rotor and adaptors, 2°C.

Fresh cells work better than frozen cells.

Estimate the pellet volume (usually ~500 µl from a 500-ml culture) and add an equal volume of ice-cold water to resuspend cells (on ice). Aliquot 50- to 300-µl cells into prechilled microcentrifuge tubes. The cell density is ~2 × 10^{11}/ml.

9b. If frozen cells are to be used for electroporation, add 40 ml ice-cold 10% glycerol to the cells and mix well. Centrifuge cells as described in step 9a.

Estimate the pellet volume and add an equal volume of ice-cold 10% glycerol to resuspend cells (on ice). Place 50- to 300-µl aliquots of cells into prechilled microcentrifuge tubes and freeze on dry ice (not in liquid nitrogen). Store at −80°C.

Prolonged incubation of cells in ice-water at all stages can increase transformation efficiency of some strains, such as BW313/P3 and MC1061/P3, >3-fold.

Transform the cells

10. Set the electroporation apparatus to 2.5 kV, 25 µF. Set the pulse controller to 200 or 400 ohms.

The pulse controller is necessary when high-voltage pulses are applied over short gaps in high-resistance samples (see Background Information).

11. Add 5 pg to 0.5 µg plasmid DNA in 1 µl to tubes containing fresh or thawed cells (on ice). Mix by tapping the tube or by swirling the cells with the pipettor.

12. Transfer the DNA and cells into a cuvette that has been chilled 5 min on ice, shake slightly to settle the cells to the bottom, and wipe the ice and water from the cuvette with a Kimwipe.

The volume of DNA added to the cells should be kept small. Adding DNA up to one-tenth of the cell volume will decrease the transformation efficiency 2- to 3-fold. Also, since the resistance of the sample should be high, make sure that addition of the DNA to the cells does not increase the total salt concentration in the cuvette by >1 mM.

13. Place the cuvette into the sample chamber.

If using a homemade apparatus, connect the electrodes to the cuvette.

14. Apply the pulse by pushing the button or flipping the switch.

15. Remove the cuvette. Immediately add 1 ml SOC medium and transfer to a sterile culture tube with a Pasteur pipet. Incubate 30 to 60 min with moderate shaking at 37°C.

If the actual voltage and time constant of the pulse are displayed on the electroporation apparatus, check this information. Verify that the set voltage was actually delivered, and record the time constant of the pulse so that you may vary it later if necessary (see Critical Parameters).

16. Plate aliquots of the transformation culture on LB plates containing antibiotics.

ALTERNATE PROTOCOL 2

DIRECT ELECTROPORETIC TRANSFER OF PLASMID DNA FROM YEAST INTO *E. COLI*

The use of "shuttle vectors"—plasmids that can be grown successfully in at least two different organisms—facilitates the transfer of DNA between, for example, yeast and *E. coli*. In this adaptation of the electroporation protocol, plasmid DNA from a shuttle vector is transformed directly from yeast into *E. coli*. Components of the interaction trap/two-hybrid system (*UNIT 20.1*) are used as an example in this protocol. The transfer and selection of a "prey" plasmid from the yeast strain EGY48 into the *E. coli* strain KC8 is described here, but the approach can be adapted for use with other yeast and *E. coli* strains.

Additional Materials *(also see Basic Protocol 2)*

Single colony of *E. coli* KC8 cells (*UNIT 20.1*)

Streak colony of Trp^- plasmid–harboring EGY48 yeast cells on Gal/Raff/Xgal/CM plates (*UNIT 20.1*), no older than 2 weeks

M9 minimal medium and plates (*UNIT 1.1*) containing 100 µg/ml ampicillin (Table 1.4.1) and standard concentrations of leucine, histidine, and uracil

Additional reagents and equipment for growth and manipulation of yeast (*UNIT 13.2*) and for plasmid DNA miniprep (*UNIT 1.6*) or PCR (*UNIT 15.1*)

1. Prepare electrocompetent KC8 cells (see Basic Protocol 2, steps 1 to 9a), resuspending the final cell pellet in ice-cold water to obtain an OD_{600} of 100.

   Fresh KC8 cells work better in this electroporation method than frozen ones.

   To measure OD, dilute 5 µl of the cell suspension with water to 1 ml and measure the OD_{600}. If necessary add more water to the suspension to get an OD_{600} of 100.

2. Distribute 65-µl aliquots of the electrocompetent *E. coli* KC8 cells into ice-cold microcentrifuge tubes.

3. With a sterile wooden or plastic stick, scrape off ~10 µl of yeast from a streak colony of EGY48 harboring the respective "prey" plasmid derivative of pJG4-5 and grown on Gal/Raff/Xgal/CM plates. Resuspend the yeast cells in the KC8 suspension by swirling the stick used for scraping off the cells.

   Avoid scraping off plate medium when collecting the yeast streak cells.

   Keep the microcentrifuge tube on ice as much as possible. Try to get an even distribution of the two cell types but do not vortex. Yeast grown on plates other than Gal/Raff/Xgal/CM will probably work as well; do not worry if the yeast colony used is blue.

4. Set the electroporation apparatus to 1.5 kV, 25 µF, and the pulse controller to 100 ohms. Transfer the cell suspension into a 0.2-cm cuvette that has been chilled 5 min on ice, shake slightly to settle the cells to the bottom, and wipe the ice and water from the cuvette with a Kimwipe.

   The use of Pasteur pipettes will facilitate placing the cell suspension at the bottom of the cuvette. Avoid any air bubbles.

5. Place the cuvette in the sample chamber of the apparatus and pulse. Take the cuvette out and place it on ice for ≥45 sec. Meanwhile, change the settings in preparation for the second pulse.

   The expected time constant for the first pulse is 2.2 to 2.4 msec.

6. Set the electroporation apparatus to 2.5 kV, 25 µF, and the pulse controller to 200 ohms. Wipe the cuvette again, place it in the sample chamber, and pulse.

   The expected time constant for the second pulse is 4.2 to 4.8 msec.

7. Remove the cuvette, immediately add 1 ml LB medium, and transfer the suspension into a microcentrifuge tube. Incubate 45 min at room temperature.

   Incubation of the suspension after electroporation for 1 hr at 37°C decreases the yield of transformants, probably due to prolonged adhesion of the E. coli cells to the yeast cell debris.

8. Spread 150 µl of the suspension evenly onto M9 minimal medium plates containing 100 µg/ml ampicillin and leucine, histidine, and uracil. Incubate ≥24 hr at 37°C.

   A slight yeast background might appear on the plates but single E. coli colonies are easy to pick. Between 50 and 200 KC8 colonies have been obtained per plate employing 150 µl out of the 1 ml LB suspension.

9. Pick a single KC8 colony, inoculate it into 1.5 to 5 ml M9 minimal medium (Leu^+, His^+, Ura^+, 100 µg/ml Amp) or LB (100 µg/ml Amp) and grow at 37°C. Harvest at an appropiate OD to prepare miniprep DNA (*UNIT 1.6*) or perform PCR analysis (*UNIT 15.1*).

   Using M9 minimal medium to grow KC8 in liquid culture is an additional safety measure but not absolutely necessary. It ensures isolation of the plasmid whose marker complements the auxotrophic defect in KC8; in addition, slightly increased plasmid copy number and improved DNA quality have been reported.

REAGENTS AND SOLUTIONS

$CaCl_2$ solution

60 mM $CaCl_2$
15% glycerol
10 mM PIPES [piperazine-*N*,*N*′-bis(2-hydroxypropanesulfonic acid)], pH 7
Filter sterilize using a disposable filter unit, or autoclave
Store at room temperature (stable for years)

SOC medium

0.5% yeast extract
2% tryptone
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM glucose
Store at room temperature (stable for years)

Transformation and storage solution (TSS), 2×

Dilute sterile (autoclaved) 40% (w/v) polyethylene glycol (PEG) 3350 to 20% PEG in sterile LB medium containing 100 mM $MgCl_2$. Add dimethyl sulfoxide (DMSO) to 10% (v/v) and adjust to pH 6.5.

COMMENTARY

Background Information

Calcium and one-step transformation

Transformation of *E. coli* was first described by Mandel and Higa (1970). Subsequent modifications to improve transformation efficiencies have included prolonged exposure of cells to $CaCl_2$ (Dagert and Ehrlich, 1974), substitution of calcium with other cations such as Rb^+ (Kushner, 1978), Mn^{2+}, and K^+, and addition of other compounds such as dimethyl sulfoxide, dithiothreitol, and cobalt hexamine chloride (Hanahan, 1983). Basic Protocol 1 given here provides good transformation efficiencies, permits long-term storage of competent cells, and is relatively uncomplicated to perform. Variations on this protocol can be obtained from the references provided. Alternate

Protocol 1, a one-step preparation and transformation procedure, is considerably faster.

Transformation by electroporation

Electroporation has become a valuable technique for transfer of nucleic acids into eukaryotic and prokaryotic cells. The method can be applied to many different *E. coli* strains and to other gram-negative and gram-positive bacteria.

In this technique, a high-voltage electric field is applied briefly to cells, apparently producing transient holes in the cell membrane through which plasmid DNA enters (Shigekawa and Dower, 1988). The field strength used for mammalian cell and plant protoplast electroporation is usually 0.5 to 1 kV/cm. The field strength needed for high-efficiency transformation of *E. coli* is much greater, usually ~12.5 kV/cm. Under these conditions up to 10^{10} transformants/μg plasmid DNA have been reported (Calvin and Hanawalt, 1988; Miller et al., 1988; Dower et al., 1988). Recently, field strength up to 8 kV/cm was also used successfully to electroporate both mammalian cells and plant protoplasts.

The capacitor discharge circuit of the electroporation apparatus typically generates an electrical pulse with an exponential decay waveform. The voltage across the electrodes rises rapidly to a peak voltage, which then declines over time as follows:

$$V_t = V_0 [e^{-t/T}]$$

where V_t = voltage at a given time t after the time of V_0, V_0 = initial voltage, t = time (sec), T = pulse time constant = RC, R = resistance of circuit (ohms); and C = capacitance of circuit (Farads).

The pulse time constant is ~5 to 10 msec for electroporating *E. coli* cells and ranges from 5 μsec to 50 msec for higher eukaryotic cells.

The pulse controller contains a number of different-sized resistors, any one of which is placed in parallel with the sample, and one resistor of fixed (20-ohm) resistance, which is placed in series with the sample. The resistor placed in parallel with the sample (usually 200 or 400 ohms) swamps out the effect of changes in the resistance of the sample on the total resistance of the circuit, thus determining the total resistance across the capacitor, and controlling the time constant (T) of the capacitor discharge. The 20-ohm resistor in series with the sample protects the circuitry by limiting the current should a short circuit (arc) occur and the capacitor discharge instantly.

The pulse controller is required when high-voltage electroporation pulses are delivered to high-resistance samples across narrow electrode gaps. In this procedure, the resistance of washed *E. coli* in the 0.2-cm cuvette is ~5000 ohms. A pulse controller is not necessary when samples of low (<1000-ohm) resistance are used, for example for electroporation of mammalian cells suspended in PBS.

Direct transfer of plasmid DNA from yeast into E. coli

Alternate Protocol 2 presents an application of electrophoretic transformation whereby a "shuttle vector" (a vector designed to be used in at least two different organisms to facilitate interspecies transfer of DNA) can be directly transferred between two species. Shuttle vectors have become increasingly popular in recent years, with those designed to facilitate the transfer of plasmid DNA between yeast and *E. coli* proving to be particularly useful. With the widespread use of the two-hybrid system (or interaction trap; see *UNIT 20.1*), transfer of plasmid DNA from yeast into *E. coli* using shuttle vectors has become a common task. As an alternative to rescuing a shuttle plasmid from a yeast clone and subsequently transforming an appropriate *E. coli* strain, the procedure described in Alternate Protocol 2 bypasses the need for plasmid isolation and offers a one-step method to obtain the same result. The direct transfer method was first mentioned by Marcil and Higgins (1992) and further modified by Karen Clemens (NIH, Bethesda, Md., pers. comm.). Alternate Protocol 2 comprises an optimization of the procedure and an adaptation to the interaction trap; however, although outlined for that specific case, the procedure is generally applicable to other yeast and *E. coli* strains. As presented, this protocol accomplishes the transfer of a "prey" plasmid from the yeast strain EGY48, designed to be used in interactor hunts, into the *E. coli* strain KC8. EGY48 contains three different plasmids (bait, prey, and lacZ reporter), all of which confer ampicillin resistance if transferred into *E. coli*. Therefore, the TRP1 selectable marker of the new interactor (prey plasmid), which complements the Trp^- phenotype of EGY48 as well as KC8, is used to rescue the plasmid. During the electroporation procedure the yeast cells are destroyed in the first pulse and the liberated plasmids transformed into *E. coli* KC8 cells in the second pulse. Selection of the prey plasmid is achieved by choosing the correct medium on which to plate the transformed KC8.

Critical Parameters

Calcium transformation

In Basic Protocol 1, preparation of competent cells with a high transformation efficiency is thought to depend on (1) harvesting bacterial cultures in logarithmic phase of growth, (2) keeping cells on ice throughout the procedure, and (3) prolonged $CaCl_2$ exposure.

At least 30 min of growth in nonselective medium (*outgrowth*) after heat shock is necessary for plasmids containing the pBR322 tetracyline resistance promoter and gene to express enough of the protein to allow the cells to form colonies with an efficiency of 1 on tetracycline plates. Cells expressing the common plasmid-encoded ampicillin resistance (β-lactamase) gene may not require such prolonged outgrowth to form colonies on ampicillin plates. When an ampicillin-resistant plasmid is used, transformation mixtures should be diluted so that transformed colonies arise at a relatively low density (≤500 cells/plate). Otherwise, the β-lactamases present in the colonies may lower the ampicillin level in the plate near them, and permit growth of weakly ampicillin-resistant *satellite colonies*. This problem can be ameliorated if carbenicillin (a related antibiotic slightly less sensitive to destruction by the pBR322 β-lactamase) is substituted for ampicillin in the medium. Carbenicillin should be used at a concentration of 50 to 100 μg/ml.

Usually only 3% to 10% of cells are competent to incorporate plasmid DNA. Transformation frequencies decrease with increasing plasmid size (Hanahan, 1983). The number of transformants obtained usually increases linearly with increasing numbers of plasmid molecules up to a point, reached at ~10 ng DNA/100 μl competent cells in the procedure given here. After this point the number of transformants does not increase linearly with increasing numbers of plasmid DNA molecules.

Transformation by electroporation

Although the procedure works with cells grown to many different densities, best results are obtained when cells are harvested at an OD_{600} of 0.5 to 0.6. After the cells are centrifuged in water, the cell pellet is very loose and the supernatant should be poured off as quickly as possible to prevent a big loss in yield; to achieve this, it is best to handle no more than two centrifuge bottles simultaneously. Fresh cells in either water or 10% to 20% glycerol usually work better than frozen cells.

As described above, the relevant parameters for exponential electroporation pulses are the time constant of the exponential curve (how long the pulse lasts) and the initial voltage or field strength (how strong the pulse is). Generally speaking, successful electroporation of *E. coli* requires long, strong pulses. In this procedure the capacitance of the circuit is relatively large (25 μF), ensuring a relatively long pulse; fine tuning of the pulse time constant is achieved by varying the size of the resistor placed in parallel with the sample.

The SOC medium must be added immediately after electroporation. Do not let the electroporated cells sit in the cuvette.

In the procedure given here, the number of transformants increases linearly with input DNA over a very wide range (from 5 pg to 500 ng). The amount of plasmid DNA added can be as little as 4 to 5 pg in 50 μl of cells, and as much as 0.5 μg in 300 μl of cells, without affecting the transformation efficiency significantly. However, transformation efficiency falls off with <5 pg of DNA; in one experiment, the number of transformants obtained with 1 pg of plasmid DNA was 30 times lower than with 5 pg.

The size of the DNA does not seem to be important for this procedure; plasmid DNA can be as large as 14 kb without significant effects on transformation efficiency. Religated DNA (still in the ligation mix) can be transformed almost as efficiently as intact supercoiled DNA.

The general recommendations given for electroporation apply to the direct transfer method as well. Although outlined for a specific case, the procedure described in Alternate Protocol 2 should be generally applicable to other yeast and *E. coli* strains. The yeast streak colony should not be older than 2 weeks. Scraping medium off the plate when collecting the yeast cells should be avoided. Fresh KC8 cells work better than frozen ones. Incubation of the 1 ml LB suspension after electroporation at 37°C for 1 hr decreases the yield of transformants, probably due to prolonged adhesion of the *E. coli* cells to the yeast cell debris. Using M9 minimal medium to grow KC8 in liquid culture is an additional safety measure, but is not absolutely necessary. It ensures isolation of the plasmid whose marker complements the auxotrophic defect in KC8; in addition slightly increased plasmid copy number and improved DNA quality have been reported.

Anticipated Results

Calcium and one-step transformation. In Basic Protocol 1, transformation efficiencies of 10^7 to 10^8 and 10^6 to 10^7/μg plasmid DNA should be obtained for *E. coli* MC1061 and DH1, respectively. In Alternate Protocol 1, transformation frequencies should range from 10^6 to 10^7 colonies/μg DNA.

Transformation by electroporation. Using Basic Protocol 2, efficiencies of $2.5–14 \times 10^{10}$ transformants/μg have been obtained with superpure pUC19 DNA (from BRL) and $6.2–12 \times 10^9$ transformants/μg with home-made pUC18 DNA and cDNA libraries in MC1061/P3. In addition, 5×10^9 transformants/μg have been obtained with the expression plasmid CDM8 in MC1061/P3. Similar results may be anticipated with MC1061 (see Table 1.4.5) and with many other commonly used lab strains. Using direct plasmid transfer from yeast to *E. coli* (Alternate Protocol 2), between 50 and 200 KC8 colonies have been obtained per plate when 150 μl out of the 1 ml LB suspension was employed.

Time Considerations

Calcium and one-step transformation. Growth of competent cells from an aliquot of an overnight culture to logarithmic phase requires ~3 hr. In Basic Protocol 1, cells are then exposed to calcium as long as overnight. Once competent cells are available, transformation requires ~90 min for either strain. In Alternate Protocol 1, preparation and transformation of competent cells requires 1 to 2 hr.

Transformation by electroporation. In Basic Protocol 2, once the culture of bacterial cells is ready to be harvested, the cells can be washed and concentrated within an hour. Electroporation takes only a few minutes, and growth and plating of the transformed cells should not take more than 90 min. The time frame for direct plasmid transfer (Alternate Protocol 2) is essentially the same as for the electroporation basic protocol. It should be noted that using M9 minimal medium slows down the growth of *E. coli*.

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Dower et al., 1988. See above.

The paper from which the second basic protocol was derived, and the highest-efficiency E. coli transformation by electroporation published to date.

Hanahan, 1983. See above.

An extremely thorough explanation of the parameters affecting transformation efficiency.

Contributed by Christine E. Seidman
(calcium) and Kevin Struhl (one-step)
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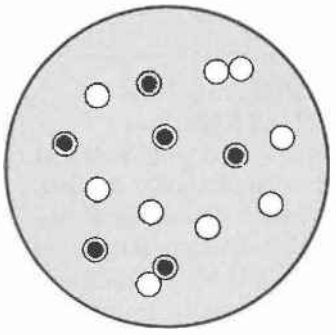
VECTORS DERIVED FROM LAMBDA AND RELATED BACTERIOPHAGES

Introduction to Lambda Phages

The biology of lambda-derived vectors is extremely well understood. This introduction to the biology of λ and related phages is included to help readers use the λ vectors that are employed in the manual (e.g., *UNIT 1.11* and Chapters 5 and 6), and to help readers understand new λ vectors that are being developed.

Lambda is a *temperate* phage, which means that it can grow lytically or lysogenically.



turbid and clear plaques

Sketch 1.9A

When λ infects a cell, it injects its DNA into the host bacterium. When it grows lytically, it makes many copies of its genome, packages them into new phage particles, and lyses the host cell to release the progeny phage. When λ grows lysogenically, it inserts its DNA into the chromosome of the host cell. The integrated phage DNA is replicated along with the host chromosome (but sometimes, e.g., when the cell's DNA is damaged, phage DNA is excised from the chromosome and the phage begins to grow lytically). Lysogens are immune to superinfection by additional λ phages carrying a homologous immunity region. Plaques of temperate phages are turbid because they contain both cells lysed by phage that have grown lytically and cells spared from further phage infection because they have become lysogens (see sketch 1.9A).

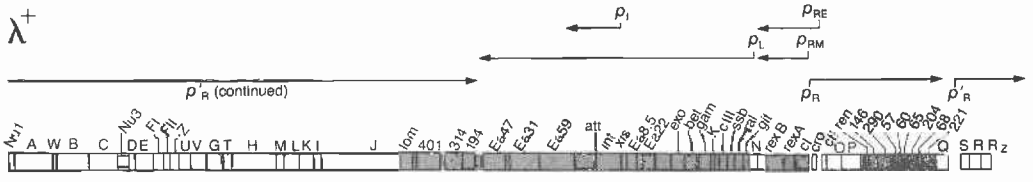
The λ genome is grouped into discrete blocks of related genes. This fact is quite convenient to the molecular biologist, as it has allowed construction of many phage-based cloning vectors which have deletions in large stretches of DNA nonessential to lytic growth (see gray areas in sketch 1.9B). In addition to these large areas, much of the immunity region, including the *cII*, *cro*, *cI* and *rex* genes, is inessential for lytic growth of otherwise wild-type phage; even the *N* gene is not essential if t_{R2} is deleted.

LYTIC GROWTH

Early Gene Expression

Lytic growth results in the production of progeny phage and the eventual lysis of the host cell. Lytic growth begins either on infection, if a phage does not grow lysogenically, or after the inactivation of existing *cI* repressor in a lysogen (see box E).

Transcription initiates from the early promoters, P_R and P_L. The P_R transcript terminates at t_{R1} and encodes the *cro* gene. The P_L transcript continues to t_{L1} and encodes the *N* gene. The product of the *N* gene is an anti-



dispensable regions of the λ genome

Sketch 1.9B

termination factor; that is, it allows the transcripts which initiate at P_L or P_R to proceed through t_{L1} and t_{R1} respectively. The *N*-antiterminated P_R transcript, which terminates at t_{R2}, encodes the *O*, *P*, and *Q* genes. The *O* and *P* proteins are required for phage DNA replication; *Q* protein is another antitermination protein, in this case, specific for transcripts initiating at a promoter located to the right of Q called P_R'. *Q*-antiterminated transcription from P_R' proceeds through another terminator called t_{R65} and the late region, then through the head and tail genes which have been joined to the same transcription unit when the incoming phage DNA circularized, and finally terminates in *b*. The P_R' transcript encodes proteins necessary for head and tail assembly, DNA packaging, and host cell lysis.

This sequential expression of phage functions allows for the replication of the λ genome, its subsequent packaging into phage heads, and lysis of the host in the correct temporal order.

A. REQUIREMENTS FOR LYTIC GROWTH

***N* antitermination factor.** Causes RNA polymerase to read through early terminators and expresses *Q* protein as well as the replication proteins.

***Q* antitermination factor.** Causes RNA polymerase to read through t_{R65} and expresses the proteins required for head and tail assembly and host cell lysis.

Replication proteins. Phage *O* and *P* proteins work together with host proteins to replicate DNA.

Packaging and lysis proteins. Other proteins essential for phage growth.

DNA Replication

Replication during lytic growth requires both host proteins and phage-encoded proteins. Lytic DNA replication can be divided into an early and late phase. The early phase begins with the injection of linear phage DNA into the bacterial cell. This DNA has, at either end, complementary, single-stranded cohesive ends generated by cleavage of the *cos* site. These sticky ends base pair, and are ligated by host enzymes to yield a covalently closed circular molecule. After supercoiling by host topoisomerases, this molecule is able to initiate DNA replication. Replication begins at a unique site (called *ori*), and proceeds bidirectionally. This type of replication results first in the formation of a theta-shaped replication intermediate and later in the production of two daughter circles.

Approximately 15 min after phage infection, replication switches to the late phase. This is characterized by *rolling-circle replication*, which produces long polymers (called *concatemers*) of repeated, full-length phage genomes. The *gam* gene product protects the concatemers from degradation by an exonuclease encoded by the host *recB* and *recC* genes. Concatemers are substrates for packaging (see below and *UNITS 5.8 & 5.9*).

B. REQUIREMENTS FOR DNA REPLICATION

***cos* ends.** 12-bp cohesive overhangs that pair and cause circularization in vivo. These are generated by cutting at *cos* sites, which occurs during packaging.

***ori*.** Site at which DNA replication starts.

***O* and *P* proteins.** *O* protein binds DNA; *P* protein interacts with host-encoded *dnaB* protein.

***gam* protein.** Inhibits *E. coli* exonuclease V (*recBC* nuclease) and thus protects the end of the rolling circle concatemer from degradation by this enzyme.

Packaging and Lysis

Late in infection, DNA replication and packaging of λ occur concurrently. Once concatemeric DNA structures are formed, they are condensed into λ proheads (incomplete head particles). *Cos* sites are recognized by λ proteins, and the DNA between two *cos* sites is cleaved from the concatemer coincident with its packaging into a single prohead (linearizing the DNA and regenerating the cohesive ends). The remaining head proteins then assemble, and the tail, which has assembled independently, attaches to the head to form the intact phage. DNA located between two *cos* sites will only be packaged in a form that can be injected if it is between 38 and 53 kb long (a fact important in choosing the proper vector to construct a library; see *UNITS 5.1* and *5.2*). The last event during lytic growth is the lysis of the host. Phage-encoded proteins disrupt the bacterial inner membrane and degrade the cell wall.

C. REQUIREMENTS FOR PACKAGING AND LYSIS

***cos* sites.** DNA is cleaved here, and DNA between the sites is packaged into phage heads.

***Nu1* and *A* proteins.** Components of the "terminase" that cleaves at *cos* sites.

***B, C, D, E,* and *Nu3* proteins.** Involved in the structure or assembly of the phage head.

***G, H, I, J, K, L, M, T, U, V,* and *Z* proteins.** Involved in the structure or assembly of the tail and tail fibers.

***R, S,* and *Rz* proteins.** *S* disrupts the inner membrane and the *R* and *Rz* proteins conspire to degrade the cell wall to achieve cellular lysis.

LYSOGENIC GROWTH

Gene Expression

Following infection, wild-type λ phage sometimes shuts down the majority of its genome and integrates into the bacterial chromosome; such a phage is called a *prophage* and the cell that contains it is called a *lysogen*. The product of the *cI* gene, λ repressor, is essential for lysogenic growth. This protein binds to the operators, O_L and O_R, blocking transcription of the early genes (from P_L and P_R) and preventing lytic growth. Binding to O_R also stimulates transcription from P_{RM}, a promoter that transcribes the *cI* gene. Lambda repressor thus maintains the lysogenic state by repressing the transcription of genes necessary for lytic growth as well as stimulating transcription of itself. There are mutant strains of *E. coli* called *hfi*$^-$, used for the construction of λgt10 libraries (*UNIT 1.11* and Chapter 5), in which a wild-type phage almost always becomes a lysogen.

The actual sequence of events leading to integration begins (as it does during lytic growth) with the joining of the cohesive ends and circularization of the genome. *cII* protein then binds to the P_{int} promoter and activates its transcription. *Int* protein is made from this transcript. *Int* protein catalyses a recombination event between the phage sequence, *attP*, and a site on the bacterial chromosome, *attB*, resulting in integration of the phage.

D. REQUIREMENTS FOR LYSOGENIZATION

***imm* region.** Contains the promoters and genes (such as *cI*) that are essential for establishment and maintenance of lysogeny (see p. 1.9.4).

***cII* and *cIII* proteins.** cII is required for int synthesis; cIII protects cII from degradation by host proteases.

***int* protein.** Along with host proteins, catalyzes the integration of the phage into the chromosome.

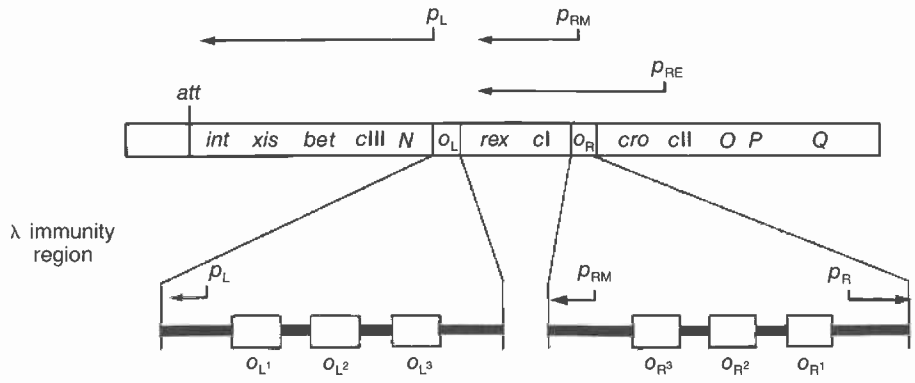
***attP* site.** Required for integration of the phage into the host chromosome.

***cos* ends.** Necessary for circularization of the molecule upon infection.

Immunity Regions

A number of bacteriophages—including 434, 21, 82, and 80—are related to phage λ, as evidenced by significant stretches of DNA homology. Unique to each phage, however, is its immunity (*imm*) region (see sketch 1.9C). This region includes a number of important regulatory sites and genes such as P_L and P_R, *cI* and *cro*, as well as O_R and O_L, and P_{RM}. A host cell stably lysogenized with a particular lambdoid phage is immune to infection by a second phage carrying the same *imm* region as the lysogen, because transcription

Sketch 1.9C



from P_L and P_R of the incoming phage is repressed. A lysogen can, however, be infected by a phage carrying a different *imm* region. This phenomenon occurs because the repressor encoded by a particular immunity region specifically recognizes and represses only its own promoters.

Induction

Lambda lysogens are *induced* when they excise from the host chromosome and undergo lytic growth. Excision requires the phage *int* and *xis* (excise) gene products. These are synthesized from the *N*-antiterminated P_L transcript. Initiation of P_L transcription occurs when the *cI* product (λ repressor) is inactivated. Induction occurs spontaneously at a low frequency in λ lysogens. However, it is more often due to (1) cleavage of λ repressor during the SOS response (see below), or (2) destabilization of a thermosensitive mutant repressor at nonpermissive temperatures.

In the laboratory, the bacterial *SOS response* is often induced by DNA damage caused by exposure of the cells to agents such as UV light or mitomycin C. During this response, LexA, a bacterial repressor protein, is inactivated and various bacterial genes are induced. Repressor proteins of λ and related bacteriophages resemble LexA and are also inactivated. Some cloning vectors contain a temperature-sensitive mutation in the *cI* gene (cI^{ts}). As a result, the repressor is stable and behaves like the wild-type repressor at 30°C, but is unstable at 42°C. cI^{ts} mutations allow lytic growth to be induced simply by increasing the temperature at which the lysogen is grown.

E. REQUIREMENTS FOR INDUCTION OF A LYSOGEN

***int* and *xis* proteins.** Necessary for the excision of the phage DNA from the host chromosome.

Lytic growth requirements. Necessary for a productive burst of phage (see box A).

E. REQUIREMENTS FOR INDUCTION OF A LYSOGEN

***int* and *xis* proteins.** Necessary for the excision of the phage DNA from the host chromosome.

Lytic growth requirements. Necessary for a productive burst of phage (see box A).

KEY REFERENCE

Hendrix, R., Roberts, J., Stahl, F., and Weisberg, R. 1983. Lambda II. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

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Boston, Massachusetts

Lambda as a Cloning Vector

ADVANTAGES OF USING LAMBDA

About the middle third of the λ genome is dispensable for lytic growth. Derivatives of phage λ that are used as cloning vectors typically contain restriction sites that flank some or all of these dispensable genes. The major advantage to using lambda-derived cloning vectors is that DNA can be inserted and packaged into phages in vitro. Although the efficiency of packaging only approaches 10%, phages, once packaged, form plaques on *E. coli* with an efficiency of 1. While techniques for transformation of bacterial plasmids have improved greatly in recent years (see UNIT 1.8), the best routinely attainable frequencies are about 10^8 transformants/μg pBR322, which means that less than 1 in 1000 plasmids become transformed into cells.

SELECTIONS FOR INSERTED DNA

Some phage vectors exploit the fact that λ has a minimum genome size requirement for packaging, so that vector phages that do not contain inserts above a minimum size are never detected. Other phage vectors utilize some genetic means to distinguish between phages that still retain the block of nonessential genes and recombinant phages in which the block of genes has been replaced with foreign DNA. Since the selection for the insert does not depend on the size of the inserted fragment, many of these vectors allow small fragments to be cloned.

Size selection. Lambda cannot be packaged into phage heads if its genome is less than 78% or more than 105% of the length of wild-type λ DNA. When a vector is chosen on the basis of size selection, the region dispensable for growth is cut out; the left and right arms of the phage are then purified and ligated to cut foreign DNA under conditions that favor concatemer formation. Those phages whose left and right arms have not been joined to an insert will have *cos* sites too close together to be packaged into viable phage. The existence of the size requirement for efficient phage packaging makes it impossible to use a single phage vector to clone fragments of all sizes: phages that can accommodate large inserts cannot be packaged if they contain small inserts, and vice versa. There are two special size selection tricks that have sometimes found use in phage cloning. In the first, packaged phage are treated with EDTA or other chelating agents. The population of surviving phage is enriched for phages with shorter than wild-type genomes. In the second, phage are plated on a mutant strain of *E. coli* called *pel*$^-$ which allows plaque formation by phages of up to 110% of wild-type length and which severely inhibits plaque formation by phages of less than wild-type length.

spi*$^-$ *selection. *red*$^+$ *gam*$^+$ phages do not form plaques on a host lysogenic for the unrelated bacteriophage P2. These phages are said to be *spi*$^+$ (sensitive to P2 interference). *red*$^-$ *gam*$^-$ phages do form plaques on P2 lysogens, and so are said to be *spi*$^-$ (Zissler et al., 1971). P2 inhibition of *red*$^+$ gam$^+$ phage growth only occurs if the P2 is wild-type for a gene called *old*. Commonly used vectors like λ2001 (Karn et al., 1984) and λEMBL3 (Frischauf et al., 1983) contain a fragment with the *red*$^+$ and *gam*$^+$ genes. These vectors will not form plaques on a P2 lysogen unless the *red*$^+$ *gam*$^+$ fragment is deleted and replaced with a piece of foreign DNA.

hfl*$^-$ *selection. The product of the λ *cII* gene is necessary for infecting phages to synthesize repressor efficiently. The *E. coli hflA* and *hflB* genes encode products whose effect is to decrease the stability of the *cII* gene product (Banuett et al., 1986; Hoyt et al., 1982). When wild-type λ infects an *hfl*$^-$ strain, so much repressor is made that the phage almost always lysogenizes the infected cell, causing it to form either no plaque or an extremely turbid plaque. Vectors like λgt10 contain a restriction site in the *cI* gene. Insertion of foreign DNA into this site inactivates the *cI* gene. The vector phage does not form plaques on the *hfl*$^-$ strain, but phages containing inserts instead of the *cI* gene form clear, normal-sized plaques.

MAPS OF LAMBDA-DERIVED CLONING VECTORS

Maps are presented for phage cloning vectors that are currently used to construct libraries, as well as maps for not-so-modern vectors which were employed to construct libraries that are still important.

The top of each page shows a simplified version of the map of wild-type lambda. The next lines show the changes that were made in the wild-type λ genome to generate that derivative. Deletions of lambda DNA are indicated by parentheses, and insertions of new DNA are indicated by bars. The bottom lines show transcripts, a genetic map, and a physical map of the resulting λ derivatives. The genetic nomenclature used, especially for deletions and changes in restriction sites, is extremely complicated. More explanation can be found in UNIT 1.9, in Lambda II by Hendrix et al. (1983), and in the articles cited in the figure legends.

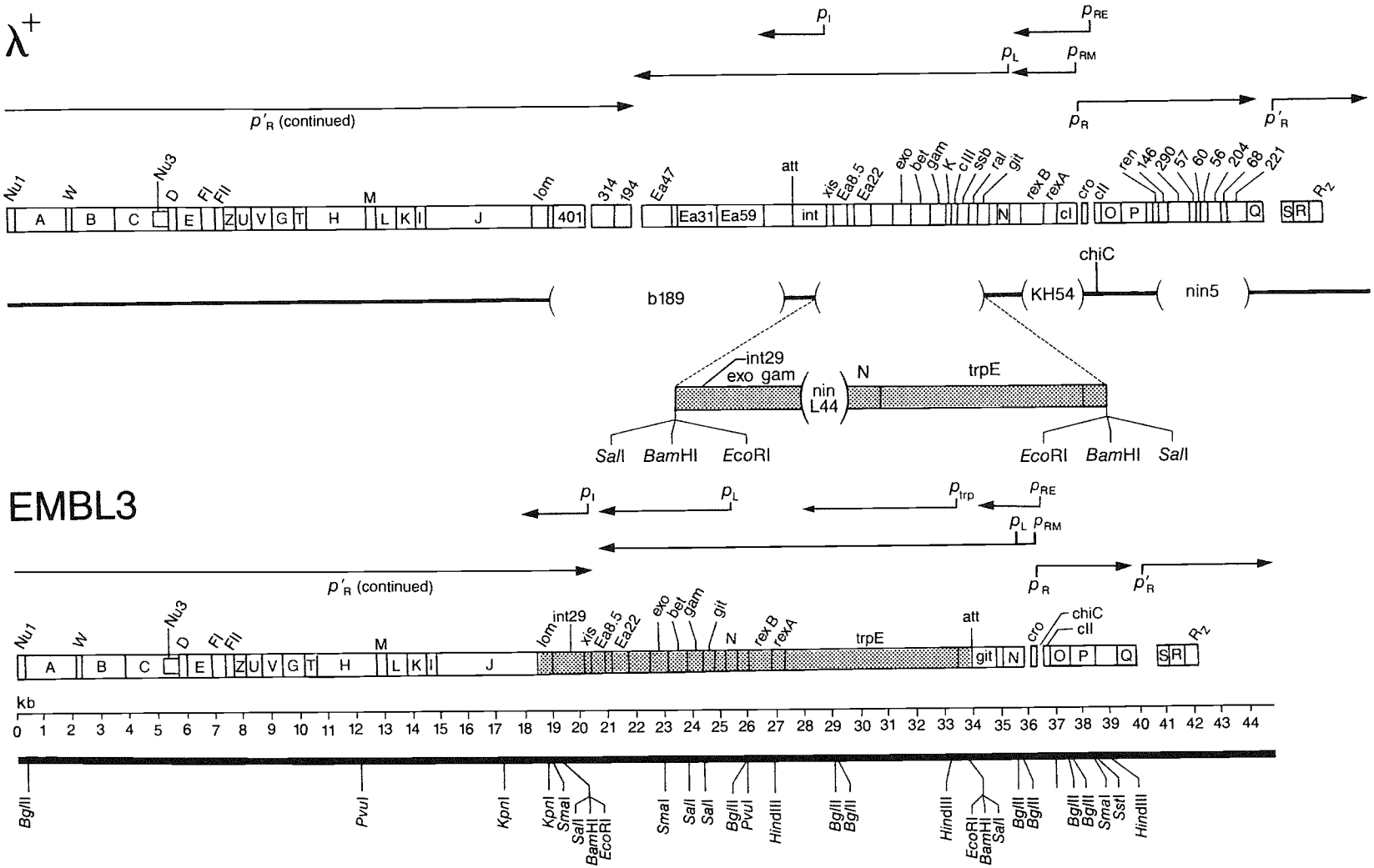


Figure 1.10.1 Wild-type lambda. Role of the major phage transcripts (arrows) and phage genes (boxes) in the lifecycle of bacteriophage λ is described in *UNITS 1.9 & 1.10.*

Figure 1.10.2 λEMBL3. This phage vector is used for cloning large (10.4 to 20 kb) fragments. It contains a polylinker with cloning sites for *Bam*HI, *Sal*I, and *Eco*RI; in EMBL4, the polylinker is reversed. Recombinant phages containing DNA inserted into these sites become *c*I⁻, gam⁻, red⁻, and *int*⁻, and thus have the Spi⁻ phenotype. Although the phage is said to carry cI857, we believe that it does not carry a *cI* gene, and it has been drawn accordingly. The polylinker sequence is GGATCTGGGTCGACGGATCCGGGGAATTCCCAGATCC. EMBL4's full genotype is λsbhIλ1° **b189** < polylinker (SalI-EcoRI) int29 ninL44 cI857 trpE polylinker (EcoRI-SalI) > KH54 chiC srIλ4° nin5 srIλ5° (Frischauff et al., 1983).

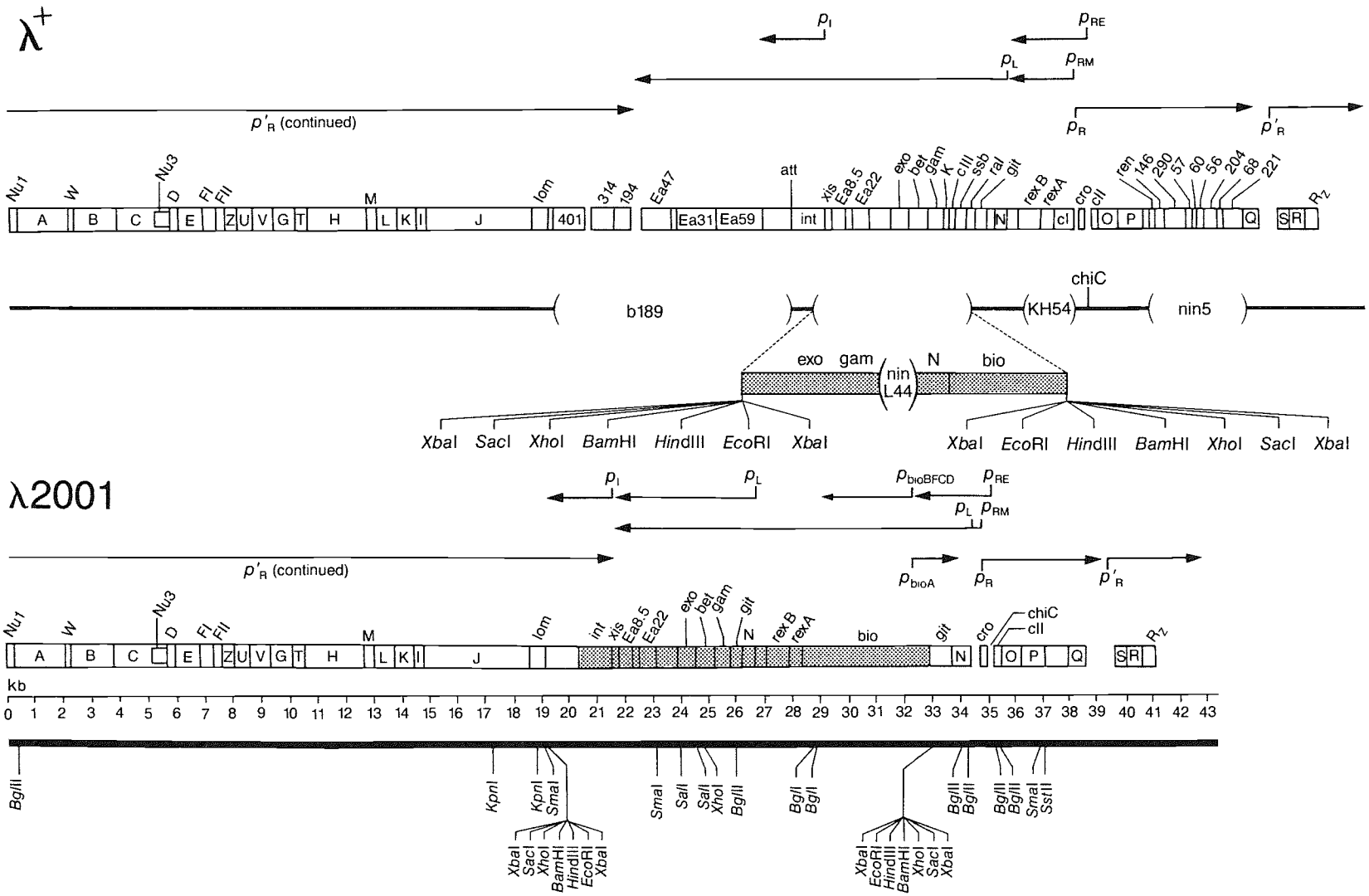


Figure 1.10.3 λ2001. This phage is used for cloning large (10.4 to 20 kb) DNA fragments. Phages containing foreign DNA become *cI*$^-$, *gam*$^-$, *int*$^-$, and *red*$^-$. Since recombinants are *red*$^-$ and *gam*$^-$, they have the Spi$^-$ phenotype, and form plaques on a strain lysogenic for bacteriophage P2 (see *UNITS 1.9* and *1.10*). The vector contains cloning sites for *Bam*HI, *Eco*RI, *Hin*dIII, *Sac*I, *Xba*I, and *Xho*I. The polylinker sequence is TCTAGAGCTCGAGGATCCAAGCTTCAATTCTAGA. λ2001's full genotype is λsbhIλ1° b189 Eint(linker) srIλ3° ninL44 EshndIIIλ4 (bio) (linker) δ(sbhIλ3-sbhIλ4) KH54 srIλ4° chiC nin5 srIλ5° shndIIIλ6° (Karn et al., 1984).

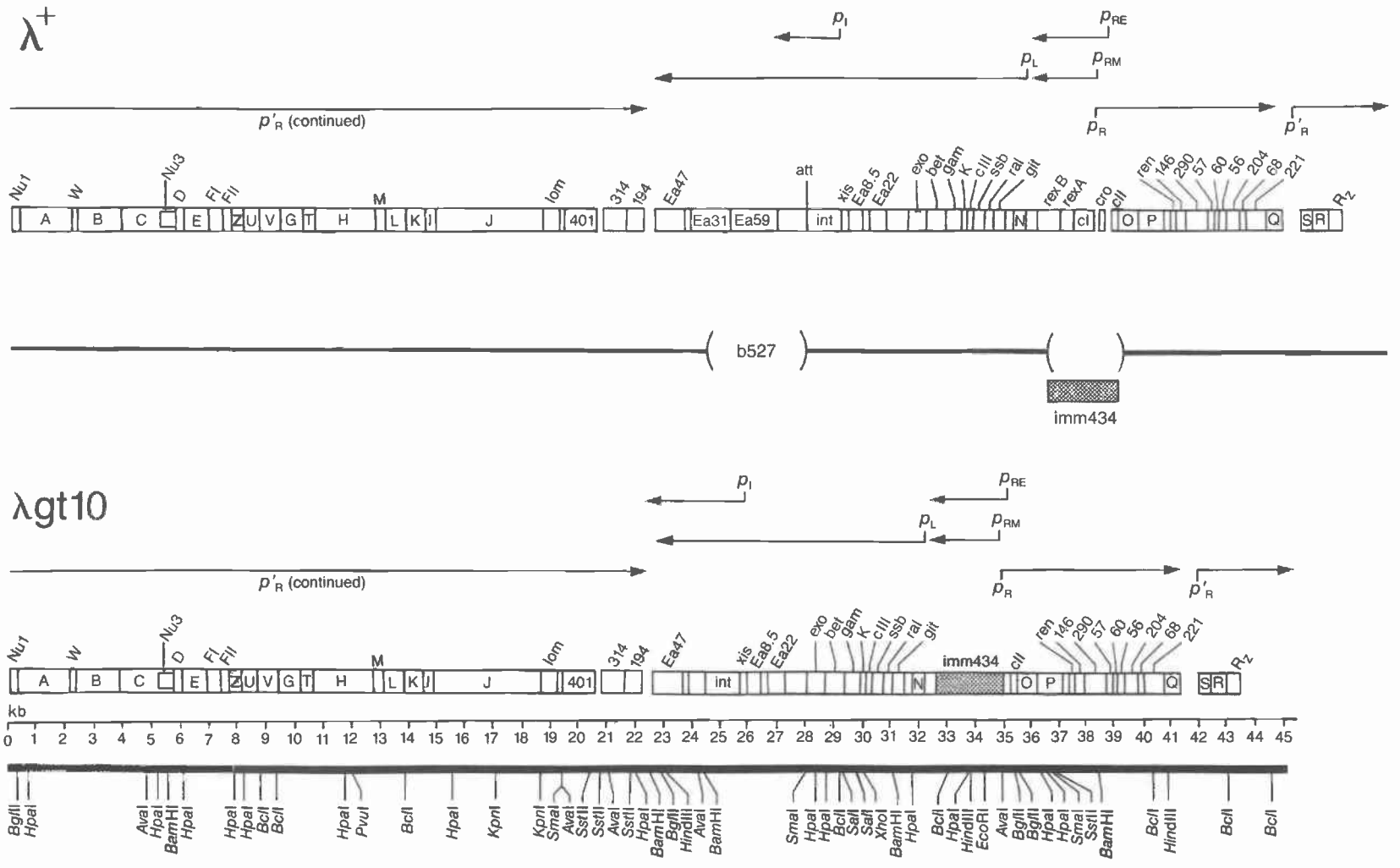


Figure 1.10.4 λgt10. This phage vector accepts small (0 to 5 kb) DNA fragments which are inserted into an RI site in its *cI* gene. Insertion of DNA into this site inactivates the *cI* gene and enables insert-containing phages to form plaques on a *hfl*⁻ host. Since insert-containing phage form plaques very efficiently on this host, and since the plaques formed by insert-containing phage are usually very healthy, λgt10 is very frequently used to construct libraries for which only very small amounts of DNA are available, for example in the construction of cDNA libraries. Insert-containing λgt10 phage are *cI*⁻ and *int*⁻ but *red*⁺. Its full genotype is λb527 srlλ3° imm^{434} srlλ 4° srlλ5° (Huynh et al., 1984).

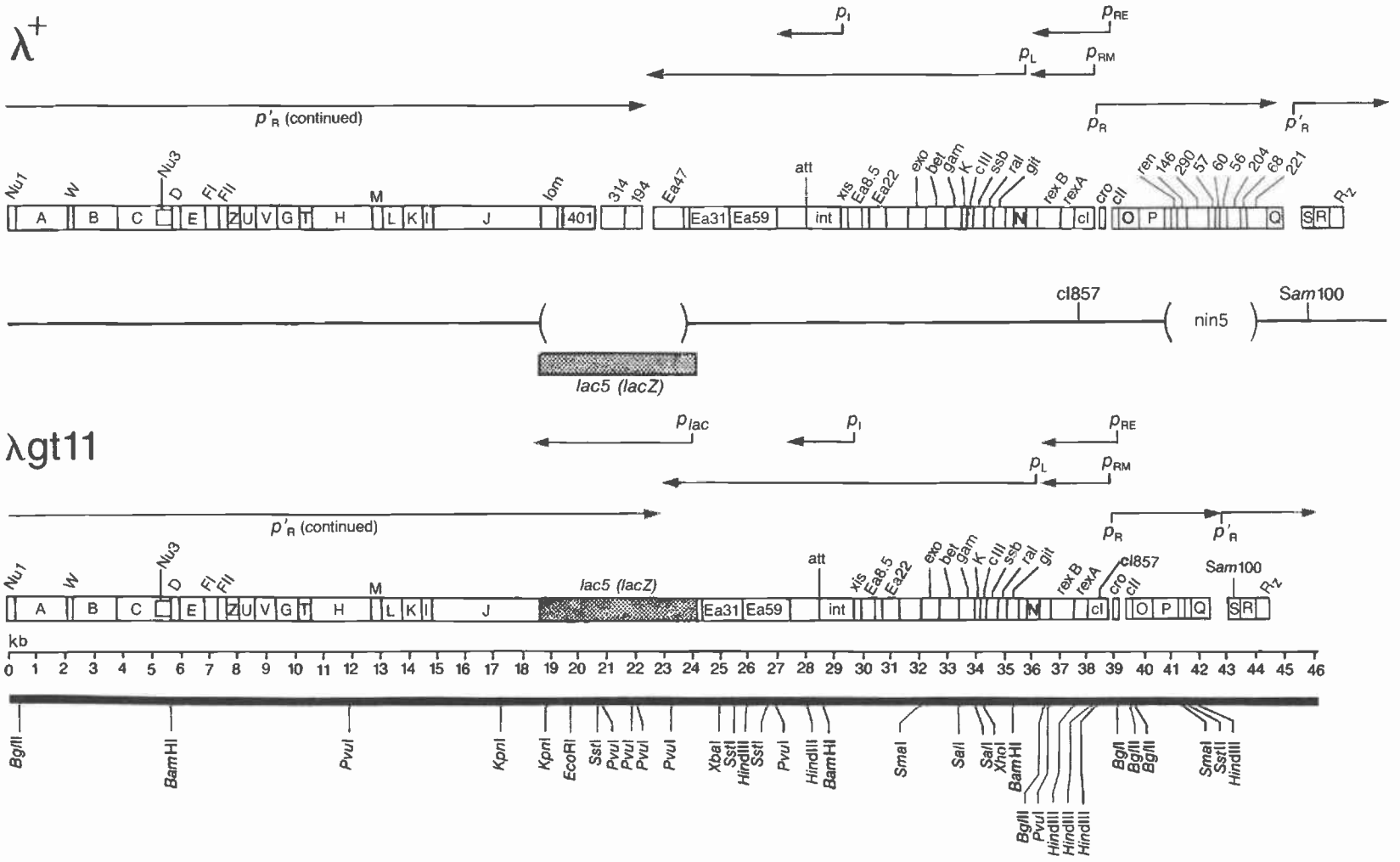


Figure 1.10.5 **λgt11.** This phage vector can accept small (0 to 4.8 kb) DNA fragments inserted into the *Eco*RI site located at the end of the *lacZ* gene. If these fragments contain a coding sequence in frame with the *lacZ* coding sequence, then the inserted DNAs are expressed in phage-infected cells as fusion proteins. Plaques made by phages encoding *lacZ* fused in frame to the coding sequence of a given protein can be identified by their ability to react with antiserum against the native protein. Recombinant phage are *cI*$^-$, *int*$^+$, and *red*$^+$. Recombinants can form lysogens from which fusion-protein production can be induced by growing the lysogen at 42°C. λgt11's full genotype is λlac5 srlλ3° cI857 srlλ4° nin5 srlλ5° Sam100 (Young and Davis, 1983).

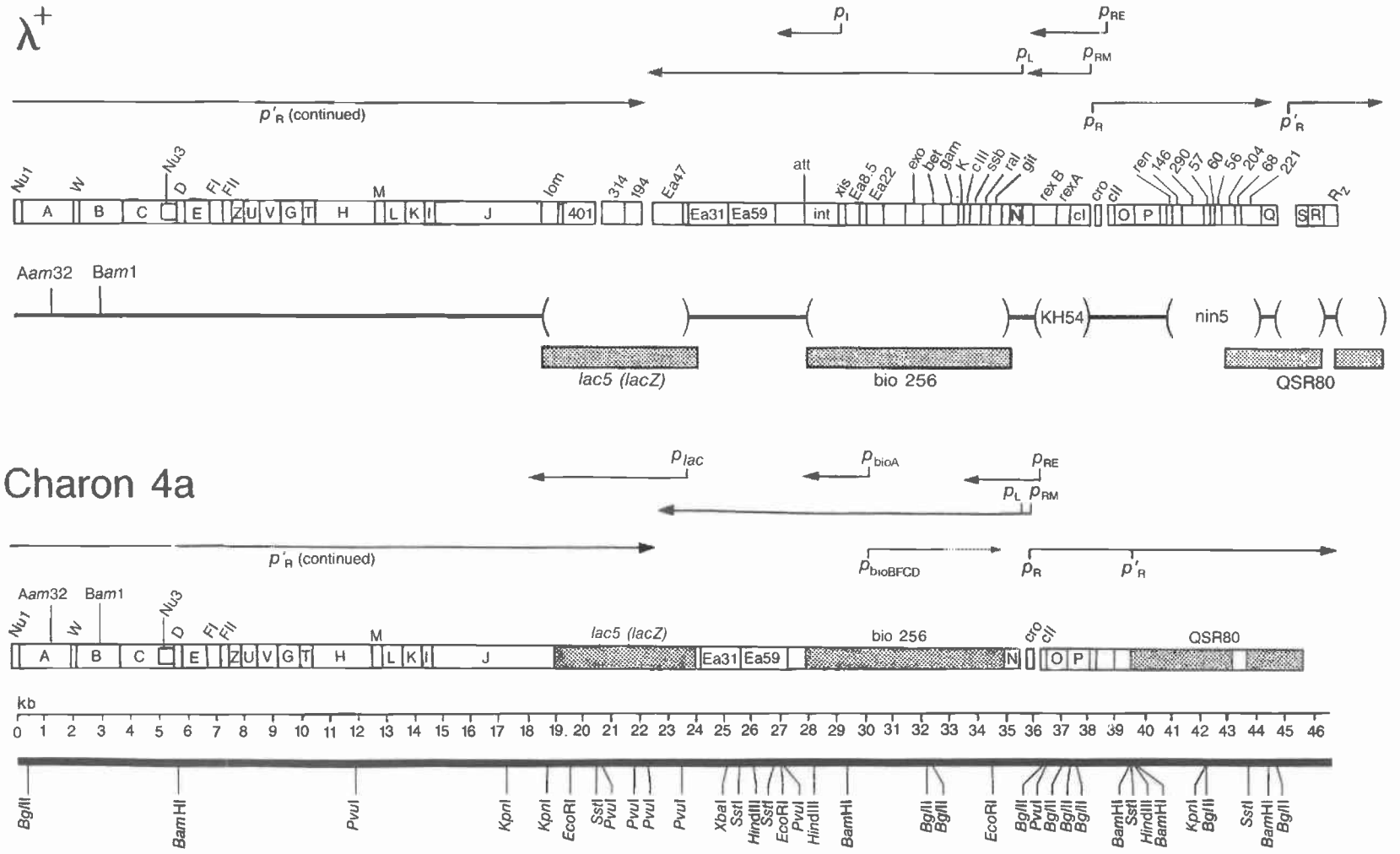


Figure 1.10.6 Charon 4a. This phage was used to construct many earlier libraries which are still being used. It contains amber mutations in the *A* and *B* genes, and so must be propagated on a host containing either *Su1* or *Su3* (see *UNIT 1.4*). It can accommodate large (7.1 to 20.1 kb) inserted *Eco*RI fragments or somewhat smaller (0 to 5.6 kb) insertions into its *Xba*I site. Recombinant phages bearing inserted *Eco*RI fragments are Bio^- and Lac^-, while phages bearing inserted *Xba*I fragments are Bio^+ and Lac^+. Its genotype is λAam32 BamI lac5 bio256 Δ*KH54 srf*λ4° nin5 QSR80 (Blattner et al., 1977; Williams and Blattner, 1979; deWet et al., 1980). The QSR80 substitution contains a short stretch of DNA from wild-type λ.

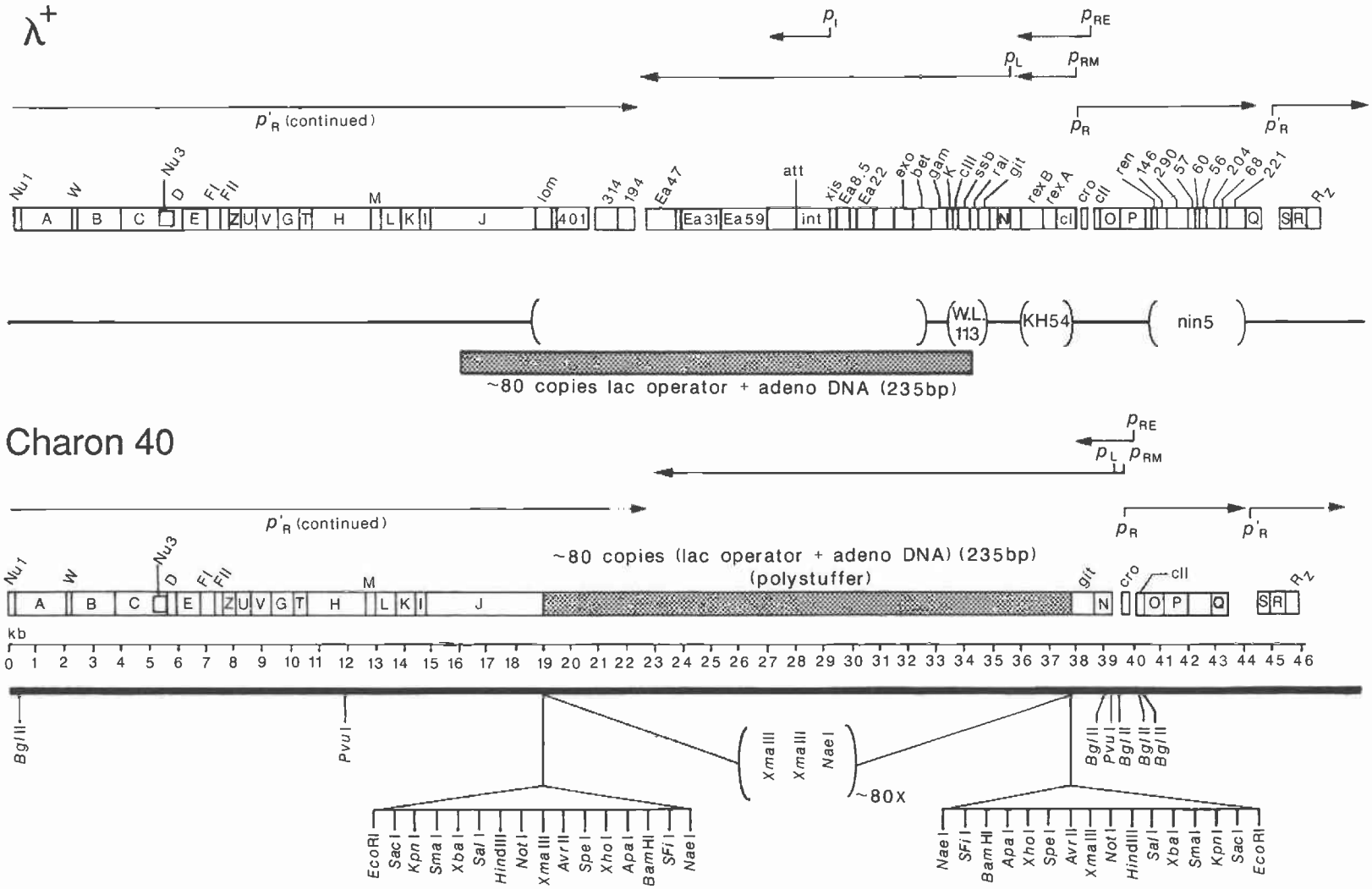


Figure 1.10.7 Charon 40. Charon 40 is a replacement vector that is useful for cloning very large (up to 24 kb) DNA fragments. The "polystuffer" is flanked by polylinkers containing 16 restriction sites including several sites that are not available in other vectors. These sites are not present in the λ arms. The polystuffer is composed of repeats of a 235-bp DNA fragment; it can be reduced to small fragments by digestion with *Nae*I. These small pieces are easily removed by polyethylene glycol precipitation. The recombinants retain *gam*, which encodes an inhibitor of the *recBC* nuclease, and thus are stably propagated even if repeated sequences are present in the insert. Even greater stability can be achieved by growth of a vector on a *recA E. coli*. Charon 40A is identical to Charon 40, except that it contains the *Aam*32 and *Bam*H1 mutations (Dunn and Blattner, 1987).

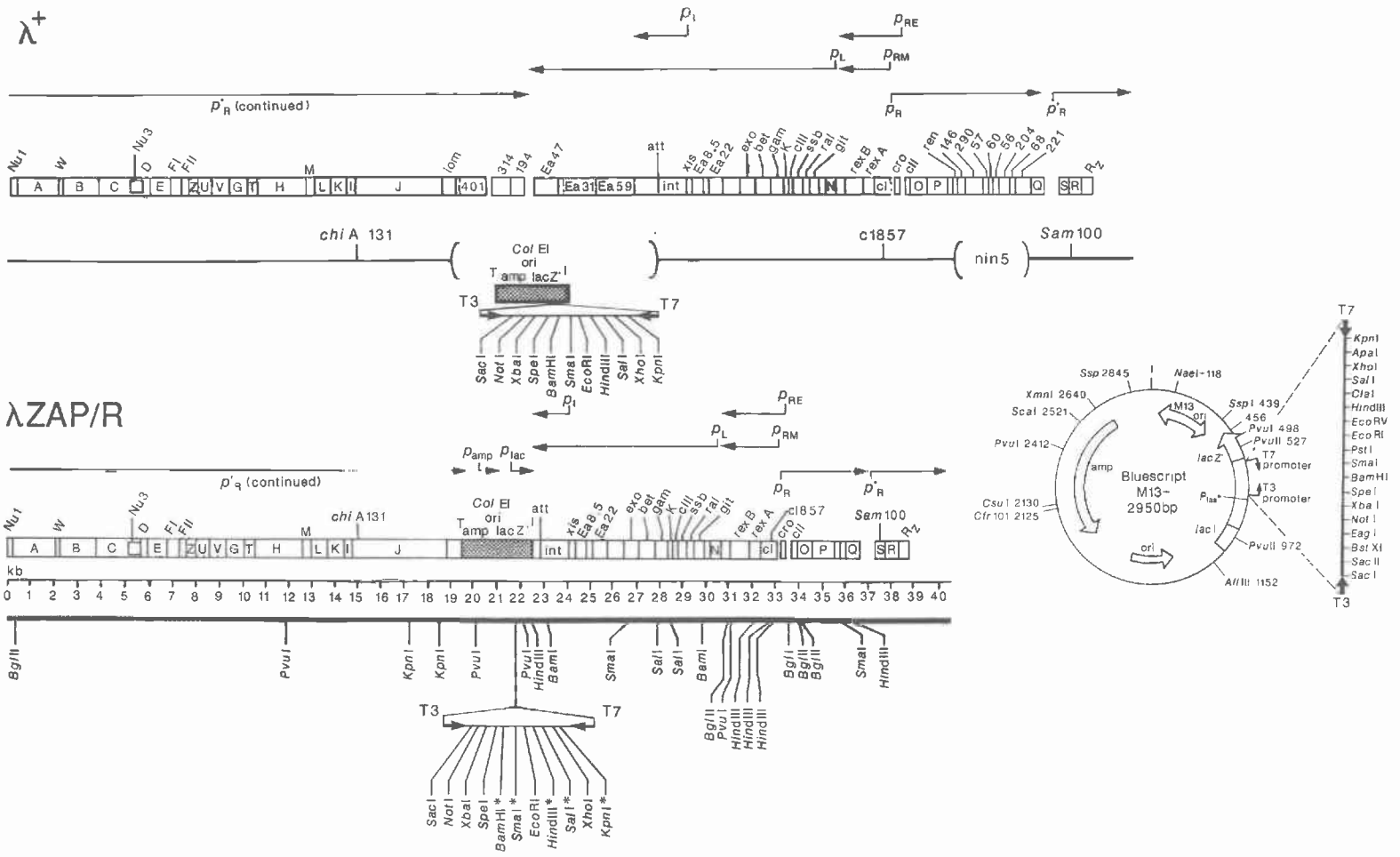
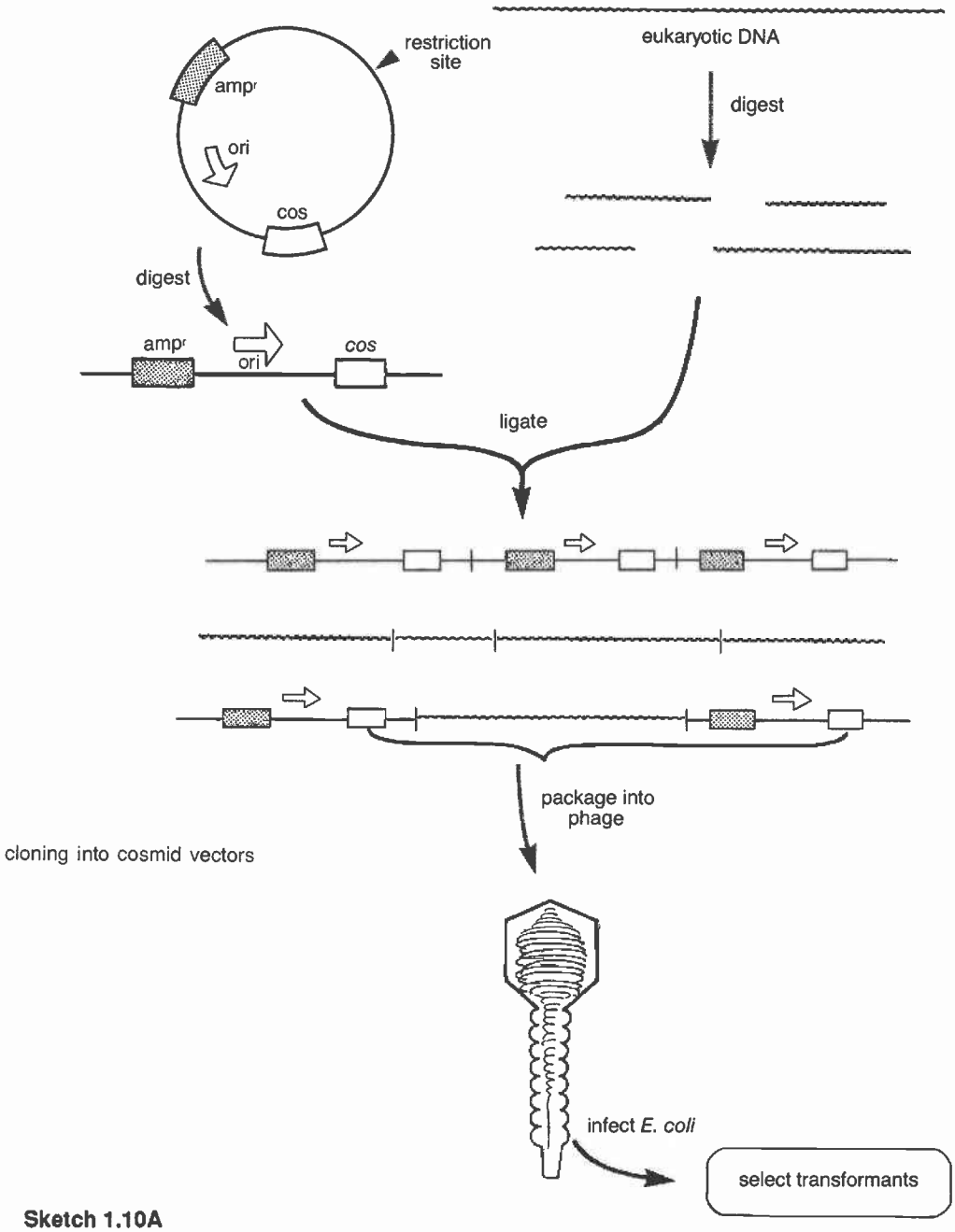


Figure 1.10.8 **λZAP.** λZAP carries pBluescript SK (–), which is excised in vivo upon infection with f1 or M13 helper phages. Inserts are cloned into λZAP within Bluescript sequences. Specifically, inserts are cloned within a polylinker located within *lacZ*. As with λgt11, a fusion protein may be expressed if the insert DNA is in frame with the *lacZ* sequence; thus, libraries made in this vector can be screened with antibodies. In λZAP, T7 and T3 promoters flank the inserts, which allows RNA probes to be easily obtained. pBluescript M13 (–), the excised plasmid (shown on the right), is normally propagated as a double-stranded circular DNA, but infection with a helper phage enables the plasmid to be propagated as single-stranded DNA. These properties facilitate sequencing of the insert, site-directed mutagenesis, and the construction of unidirectional deletions. DNA fragments up to 10 kb can be inserted. Within the polylinker, unique *Xho*I, *Eco*RI, *Spe*I, *Xba*I, *Not*I, and *Sac*I cloning sites are available. λZAP/R is shown in the figure. λZAP/L is identical to λZAP/R except that the polylinker is inverted. λZAP vectors are available from Stratagene.

THE COSMID, A USEFUL LAMBDA-DERIVED PLASMID VECTOR

Cosmids were developed to allow cloning of large pieces of DNA. How they work is diagrammed in sketch 1.10A. Cosmids contain a selectable marker, a plasmid origin of replication, a site into which DNA can be inserted, and a *cos* site from phage λ. The vector is cut with a restriction enzyme and mixed with pieces of DNA to be cloned. DNA ligase joins cut vector and insert fragments into concatemeric molecules. The ligation mixture is then mixed with a *packaging extract* (see UNIT 1.11) containing the proteins necessary to package naked phage DNA into phage heads. Whenever two *cos* sites are present on a concatemer and separated by 40,000 to 50,000 nucleotides, they will be cut and packaged into phage heads. The cosmid-containing phages are infectious, they inject DNA into cells, but the DNA is plasmid DNA: the phage adsorbs to the host and the cosmid DNA is injected into the cell, which circularizes due to its sticky ends. The annealed ends are then covalently joined by the host's ligase, and the resulting large circular molecule replicates as a plasmid (see map, Fig. 1.10.9).



Sketch 1.10A

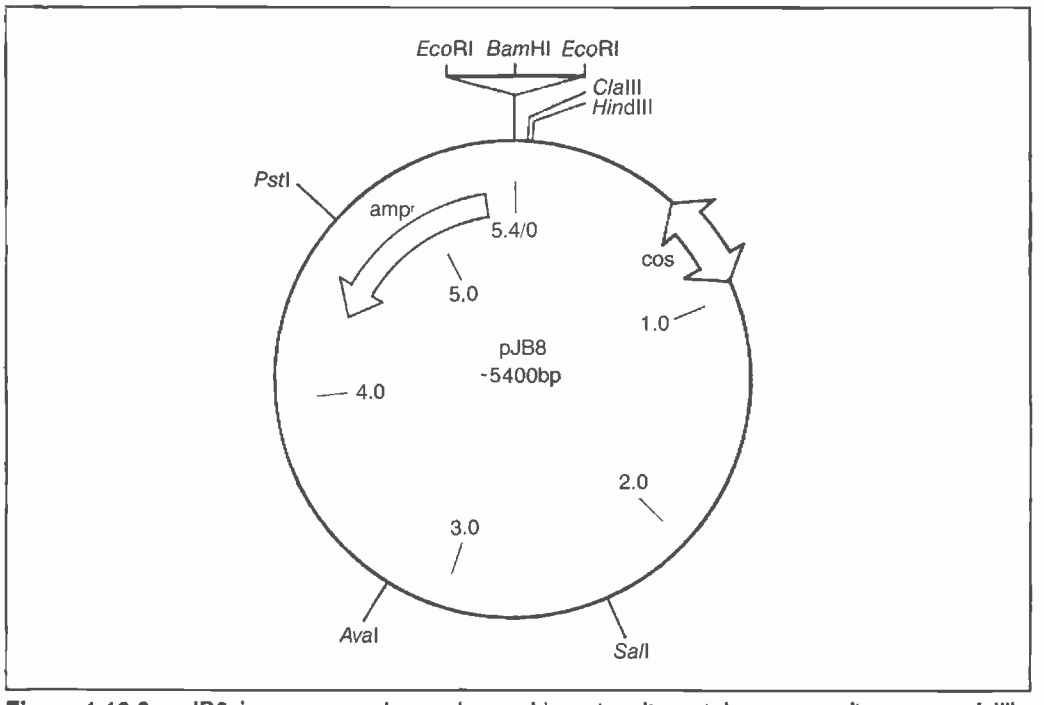


Figure 1.10.9 pJB8 is a commonly used cosmid vector. It contains a *cos* site, an ampicillin resistance gene, and a pMB1 replicator, so it can be amplified with chloramphenicol. In a typical application, libraries are constructed by insertion of random *Sau*3a partial-digestion fragments of DNA into its *Bam*HI site. The inserts can be excised by cleaving the plasmid at its flanking *Eco*RI sites (Ish-Horowitz and Burke, 1981).

Cosmid vectors within cells replicate using their pBR322 origins. Intracellular cosmids sometimes rearrange DNA inserted into them, perhaps because the time it takes pBR322-dependent replication to replicate the 50,000-bp cosmid is almost as long as the generation time of *E. coli*, so that cosmids which have deleted sections of DNA have a growth advantage on antibiotic-containing medium. Although this problem can be ameliorated by propagating the cosmids in cells that are less prone to rearrange vector DNA (*UNIT 1.4*), another approach may well become more popular. Cosmid vectors (called *lorist vectors*) have recently been developed that are said to circumvent this problem by using the λ *ori* and *O* and *P* proteins to replicate (see, for example, Gibson et al., 1987). It takes only a few minutes for these vectors to replicate inside cells, and insertions in them are said to be more stable.

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Provides a thorough introduction to the biology of cloning vectors in common use before 1983.

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Nina Irwin
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Plating Lambda Phage to Generate Plaques

BASIC PROTOCOL

ISOLATING A SINGLE PLAQUE BY TITERING SERIAL DILUTIONS

This procedure is used to isolate pure populations of phage from a single plaque and provide the titer of the phage stock. Serial dilutions are made of a phage lysate. In separate tubes, aliquots of each dilution are mixed with *E. coli*. Phage are allowed to adsorb to the cells and the cell/phage mixture is then heated to 37°C, causing the phage to inject their DNA into the cells. Top agar is added to each tube, and the mixture is poured onto rich plates, which are incubated at 37°C until plaques appear. Each plaque contains phages derived from a single infecting phage.

Materials

Lambda broth (*UNIT 1.1*)
0.2% maltose
10 mM $MgSO_4$
Lambda top agar (*UNIT 1.1*)
Suspension medium (SM)
Fresh lambda plates (*UNIT 1.1*), prewarmed to 37°C
Microwave oven or boiling water bath
45° to 50°C water bath
8 × 80–mm tubes
37°C water bath or heat block
Capillary tubes or toothpicks

NOTE: All materials coming into contact with *E. coli* must be sterile.

1. Grow a culture of *E. coli* to saturation in lambda broth + 0.2% maltose + 10 mM $MgSO_4$.

   Growth in maltose induces production in E. coli of the λ receptor (lamB protein), which is necessary for maltose transport. Mg^{++} ions also aid phage adsorbtion.

2. Melt top agar in microwave oven set to defrost setting, or in boiling water bath for 15 min. After agar is melted, let bottle cool at room temperature for 5 min, then place bottle of melted agar in 45° to 50°C water bath.

   The cap to the bottle of top agar must be loose before putting it in microwave! Watch the microwave oven to make sure that the contents of the bottle of top agar do not boil over. If agar boils over, carefully take the bottle out and swirl it around to see if there are any unmelted flecks of agar. If there are, reinsert into oven and microwave longer, inspecting occasionally, until the agar is completely melted.

   Be sure top agar is left in the water bath enough time to cool to 45° to 50°C. Cells will be killed by even brief exposure to agar that is hotter than 65°C.

3. Add 0.3 ml of the *E. coli* culture to five 8 × 80–mm tubes.

4. Make serial dilutions of the phage lysate in SM (see *UNIT 1.3* for serial dilutions).

   Dilution factors of 100-fold are usually used. Label the dilution tubes so that they do not get mixed up.

5. Add 0.1 ml of the first dilution to one tube of *E. coli*, 0.1 ml of the next dilution to the next tube, etc. Label the tubes of *E. coli*/phage mixture, so that they do not get mixed up. Incubate tubes at room temperature for 20 min.

   The phage adsorb to the E. coli during this step.

6. Move tubes to a 37°C water bath or heat block for 10 min. While tubes are in

water bath, label 5 fresh, prewarmed lambda plates to correspond with the labels on the dilution tubes.

During this step, the phage inject their DNA into the cells.

Plates should be fresh, but not so fresh that they are wet on their surfaces, nor so wet that they will exude moisture and cause the lawn of cells in top agar to slide away.

7. Remove tubes from the water bath. Add 2.5 ml top agar to one tube, vortex lightly to mix, and pour the contents of tube onto a plate. Spread agar over the entire surface of the plate by tilting it gently.
8. Place the plates in a 37°C incubator. Plaques of lambda-derived phages will appear after 6 to 8 hr, but will be easier to score, count, and pick if left for 12 hr.
9. From one of the dilution plates that is not too crowded with plaques, pick a single plaque with a sterile capillary tube or toothpick. To save the plaque for future use (for example, to make a plate stock), cut out a plug of agar containing the plaque with a capillary tube, and blow the plug into a tube containing 1 ml of SM (or place the tip of the toothpick in the liquid and agitate gently). If desired, count the number of plaques on one of the dilution plates and use this number to compute the number of viable phage in the starting stock.

Background Information

Titration of bacteriophage, and isolation of phage from single plaques, was first described by d'Herelle in 1920. The best general background to bacteriophage growth protocols is probably found in Stent (1971).

Genes encoding the tail proteins of most lambda-derived cloning vectors come from phage λ. Vectors with these tail proteins, said to be "hλ" (for host range of λ), adsorb to the cell *lamB* protein, which is involved in maltose uptake. These vectors make plaques with sharp boundaries. Some vectors have tail proteins derived from phage 80 and are said to be "h80." These vectors adsorb to the host *tonA* protein, which is involved in ion transport. They make slightly larger plaques with fuzzier borders than those made by hλ phages. Stocks of h80 phages usually have a higher titer than stocks of corresponding hλ phages.

Troubleshooting

It is sometimes helpful to include two other control plates in the procedure. One is a plate that contains a lawn made from a separate tube of the *E. coli* culture that has not been infected with phage. The other is a plate that contains top agar that did not contain any cells. These two controls provide benchmarks for growth of the lawn, as it becomes denser during the time in the incubator. If the lawn appears crinkled, then the top agar/*E. coli*/phage mixture was probably too cold by the time it was poured onto the plate. If this occurs, try warming the plates up to 37°C before pouring the lawn onto them, or pouring the lawns more quickly after the top agar is added to the *E. coli*/phage mixture. If the top agar layer floats off the plate, then the plates were too wet. If this occurs, use dryer plates.

Time Considerations

It takes from 6 to 8 hr of incubation at 37°C before plaques appear, and is often 12 to 14 hr before differences in morphology can be reliably distinguished.

BASIC PROTOCOL

ISOLATING SINGLE PLAQUES BY STREAKING ON A LAWN OF CELLS

Phages are streaked for single plaques on a plate containing a prepoured lawn of *E. coli*. This procedure is easier than titering by serial dilutions, and is recommended if only a few isolated plaques are needed.

Materials

LB medium (*UNIT 1.1*)
Lambda top agar (*UNIT 1.1*)
Rich plate (*UNIT 1.1*), prewarmed to 37°C
32-G platinum wire loop or sterile 1½ × ⅛ in. strips of paper

1. Grow a lambda-sensitive strain of *E. coli* to saturation in 5 ml LB medium.
2. Add 0.2 ml of the saturated culture to 2 ml of melted top agar (cooled to about 45°C; see step 2, p. 1.11.1), and pour evenly over the top of a prewarmed, rich plate.

   In the recipe for top agar (UNIT 1.1), agar can be replaced with 6 g agarose.
3. After top agar has hardened, cool plates by placing in refrigerator ≥15 min.
4. Spot 100 µl of λ stock culture (usually around 10^8 phage/ml) on the corner of the plate.
5. Using the techniques described in *UNIT 1.3* to streak out single bacterial colonies, *lightly* streak out the phage using a thin wire loop or the edge of a sterile 1½ × ⅛ in. piece of paper.

   Paper should be cut into strips and sterilized by autoclaving (dry) in screw-cap vials.

BASIC PROTOCOL

PHAGE TRANSFECTION AND IN VITRO PACKAGING

Construction of a library with lambda-derived cloning vectors results in a population of phage DNA molecules. In order for these molecules to be replicated, they must be introduced into cells so that they can grow as phage. Phage DNA is typically introduced into cells either by infection after packaging into phage particles in vitro or, much less frequently, by *transfection*, that is, transformation of phage DNA that has been circularized by treatment with DNA ligase into competent cells.

In vitro packaging uses lysates of phage-infected *E. coli* called *packaging extracts*. These lysates contain empty phage heads, unattached phage tails, and the phage-encoded proteins required for DNA packaging (see *UNIT 1.9*). If ATP is present, then concatemeric phage DNA mixed with the extract is cut at one *cos* site by the terminase (probably a complex of the *A* and *Nul* proteins) and loaded into the phage head by an unknown mechanism. DNA continues to be loaded into the phage head until the terminase encounters and cuts the next *cos* site on the molecule. The phage tails then attach themselves to the filled heads.

In the early 1980s, production of high-efficiency packaging extracts was considered one of the biggest inconveniences in library construction. To avoid the work involved, most researchers now use frozen packaging extracts purchased from commercial suppliers (see Enquist and Sternberg, 1979). Commercial extracts typically yield 2×10^8 to 2×10^9 plaque-forming particles per µg of concatemerized phage DNA. Phage DNA can also be introduced into *E. coli* by transformation of competent cells. In this procedure, linear or circular phage DNA is mixed with competent *E. coli*, and the mixture of cells and DNA is treated as in *UNIT 1.8*. After the heat shock step, the calcium-treated cells are mixed with *E. coli* that have been grown in lambda broth and maltose as described on p. 1.11.1. Molten top agar is then added, the mixture is poured

onto a lambda plate, allowed to solidify, and incubated at 37°C until plaques appear. This procedure typically yields 10^4 plaques/μg of phage DNA.

REAGENTS AND SOLUTIONS

Suspension medium (SM), per liter

5.8 g NaCl
2 g $MgSO_4 \cdot 7H_2O$
50 ml 1 M Tris·Cl, pH 7.5
0.01% gelatin (Difco)

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Growing Lambda-Derived Vectors

It is often necessary to grow large quantities of lambda-derived vectors, so that DNA can be made from them (see *UNIT 1.14*). The following basic protocol tells how to make a phage stock by plate lysis, while the alternate protocol tells how to make a liquid lysate. Storage of phage lysates is described in the support protocol.

BASIC PROTOCOL

MAKING A STOCK OF PHAGE BY PLATE LYSIS

Phage from a single plaque are mixed with cells and top agar and poured onto a plate. Although there are initially more cells than phage in the lawn, the phage increase their number more rapidly than the cells, and eventually lyse most cells on the plate. The top agar is then scraped off the plate and the phage in it are extracted and saved.

Materials

Fresh lambda top agar (*UNIT 1.1*)
Fresh lambda plates (*UNIT 1.1*), prewarmed to 37°C
Suspension medium (SM; *UNIT 1.11*)
Chloroform
45° to 50°C water bath
Capillary tube or toothpick
Beckman JA-21 rotor or equivalent

NOTE: All materials coming into contact with *E. coli* must be sterile.

1. Dilute a fresh overnight culture of a lambda-sensitive strain of *E. coli* 50-fold and grow in culture tube on a roller drum at 37°C.
2. Melt 100-ml bottle of fresh lambda top agar by heating in microwave oven set to defrost. Place in 45° to 50°C water bath.
3. When cell density reaches 2 to 3×10^8/ml ($OD_{600} = 0.4$), place 0.75 to 1 ml of cells into a test tube. Pick a single fresh plaque with capillary tube or toothpick, blow the plug into cells, and vortex lightly for 10 sec.
4. Add 7.5 ml top agar, vortex gently, and pour equal amounts of the mixture onto three prewarmed fresh lambda plates.

   If agar in the top agar and plate recipes is replaced by agarose, the extractable DNA will be an acceptable substrate for restriction enzymes and ligases.

5. Incubate plates at 37°C, typically for 4 to 6 hr, until plaques are clearly visible and when 90% to 100% of the lawn is lysed.
6. Pipet 3 ml SM onto plate. Using clean microscope slide, scrape top agar from all 3 plates into a small centrifuge tube (e.g., a 15-ml screw-top tube for the Beckman JA-21 rotor).
7. Add 3 drops chloroform. Vortex vigorously for 10 sec.
8. Leave at room temperature 10 min.

   As an alternative to steps 6 through 8, 3 ml SM and 3 drops chloroform can be dribbled onto the plate, the plate left overnight at 4°C, and the liquid decanted and centrifuged as below.

9. Centrifuge 10 min at 10,000 rpm in JA-21 rotor ($11,400 \times g$), 4°C.
10. Gently decant and save supernatant.

MAKING A LIQUID LYSATE

ALTERNATE PROTOCOL

Host bacteria grown to saturation are infected with 10^5 to 10^8 phage/ml. Following phage adsorption, the infection mixture is diluted into a rich medium and shaken vigorously until cell lysis. Any remaining viable cells are lysed with chloroform, and cell debris is removed with a low-speed spin.

Additional Materials

LB medium (*UNIT 1.1*)
Lambda dilution buffer
10 mM $MgCl_2$/10 mM $CaCl_2$
NZC medium (*UNIT 1.1*)
Beckman JA-20 rotor or equivalent

1. Grow an overnight culture of a lambda-sensitive strain of *E. coli* in LB medium at 37°C.

   Lambda-sensitive strains will support lytic growth. This can be tested by spotting 10 µl of a lambda lysate onto a lawn of bacteria. If the strain is lambda-sensitive, a plaque will form where the phage were spotted.

2. Using a sterile toothpick or capillary tube, pick a single plaque (see step 3 of basic protocol), blow it into a tube that contains 0.4 ml lambda dilution buffer, and place tube at 4°C for 2 hr to allow phage to elute.

   Alternatively, 10^5 to 10^8 phage from a liquid lysate or plate stock can be used.

3. Combine 0.1 ml eluted phage with 0.1 ml of saturated culture and 0.1 ml of 10 mM $MgCl_2$/10 mM $CaCl_2$ solution and incubate 15 min in a 37°C water bath.

   Incubating with Mg^{++} and Ca^{++} allows the phage to adsorb to the bacteria.

4. Transfer this solution to 50 ml of NZC medium and shake *vigorously* at 37°C until lysis occurs (usually between 6 and 8 hr).

   Good aeration is important for high yields.

5. The culture should be checked frequently after 6 hr, and harvested immediately upon clearing.

6. Add a few drops of chloroform to lyse any remaining cells, transfer the solution to Co-rex or Nalgene tubes (being careful to leave the chloroform behind), and spin 10 min at 10,000 rpm (12,100 × *g*), 4°C, to pellet the cell debris.

7. Save as much of the lysate as desired. Transfer to a screw-cap tube, add a few drops of chloroform, vortex briefly, and store at 4°C.

   The titer of the phage should be determined as described in UNIT 1.11.

STORING PHAGE LYSATES

SUPPORT PROTOCOL

Phage stocks should be stored in SM (*UNIT 1.11*) plus a few drops of chloroform at 4°C. Screw-cap glass tubes with rubber-lined or teflon caps, capable of holding at least 1 ml, are generally used, although disposable plastic tubes work well. Lambda titers drop over a period of several years. Stocks should be dated and checked occasionally to determine phage titer. Addition of 0.1% gelatin to the SM slows down the rate at which phage titers drop when stored under these conditions. Lysates can also be stored frozen in 15% glycerol.

REAGENTS AND SOLUTIONS

Lambda dilution buffer

20 mM Tris·Cl, pH 8
20 mM $MgCl_2$

COMMENTARY

Background Information

Plate lysis is typically used to make small phage stocks, which are then used as intermediates for making larger stocks for preparation of phage DNA. However, if agar is replaced by agarose in the plate recipe and in the top agar (see *UNIT 1.1*), then DNA extracted from phage in the lysate is an acceptable substrate for restriction enzymes and ligases.

Liquid lysates generally give slightly lower yields than plate lysates. Less work is required, however, especially when large quantities of phage are needed. This fact, taken together with the fact that there is no contaminating agar in the lysate, makes this method preferable for isolating phage for DNA preps. In the liquid lysis procedure, cells are diluted into NZC medium after phage adsorption. This is a rich medium that contains amino acids but no glucose or other sugars. In cells grown in this medium, sugar receptors like LamB protein are fractionally induced (so phage can adsorb to the cell), but cells are not covered with so many receptors that adsorption to their debris depletes the yield of phage from the lysate. In addition, cells grown on this medium seem to cause infecting phage to grow lytically, which helps to obtain high titers of turbid phages.

Critical Parameters

LB medium and top agar can replace lambda plates and top agar for the plate lysis method, but the phage yield is usually lower. It is important that the plates be freshly poured, and that the top agar be freshly melted. The plates do not even have to be dry; a loose lawn is no problem as long as the plates are not tilted. If the yield is less than expected, it may be because the plates were not fresh, or because the particular phage used does not give a burst as large as that given by wild-type lambda. The size of single plaques made by the phage sometimes gives a clue that the second problem obtains. If plaques made by the phage in question are smaller than those made by closely related phages, it is often worthwhile to reduce the number of starting cells, or to increase the starting number of phage by combining several plaques, and to incubate the plates longer until the lawn is completely lysed.

In the liquid lysis method, good aeration is essential for high yields. Flasks should be no more than ⅕ full. Cultures should be watched carefully as they approach lysis and harvested soon after lysis is complete to prevent released phage from reinfecting cell debris. Lysis usually takes between 6 and 8 hr. If there is no sign of lysis after 8 hr, there probably will not be any, and one can either go on to add the chloroform and complete the procedure, or abandon hope and start over.

Anticipated Results

The plate lysis procedure yields ~10 ml of a phage stock containing 10^{10} to 10^{11} phage/ml. The liquid lysis procedure usually yields a stock with 5×10^9 to 3×10^{10} phage/ml.

Time Considerations

The plate lysate procedure usually takes about 8 hr. The liquid lysate procedure takes 2 hr to elute the phage from the plaque, and 6 to 9 hr between the time the cells are infected and the time the lysate is harvested. The time required to elute the phage is very flexible. Two hours will ensure that more than 90% of the phages are eluted from the plaque; shorter elution times allow less phage to be eluted. Elution times as short as 30 min generally work but do not always give good lysis. Phage may be eluted for longer periods of time; overnight is often convenient. Once the host cells are infected, it takes ~6 to 9 hr for lysis and preparation of the lysate.

Key Reference

Blattner, F.R., Williams, B.G., Blechl, A.E., Denniston-Thompson, K., Faber, H.E., Furlong, L.-A., Grunwald, D.J., Kiefer, D.O., Moore, D.D., Schumm, J.W., Sheldon, E.O., and Smithies, O. 1977. Charon phages: Safer derivatives of bacteriophage lambda for DNA cloning. *Science* 196:161-169.

Origin of this liquid lysate procedure.

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Preparing Lambda DNA from Phage Lysates

DNA extracted from lambda-derived vectors is typically subcloned into plasmid or filamentous phage vectors. The first two protocols describe methods for isolating phage DNA from large- and medium-scale liquid lysates. These two methods use either density-gradient centrifugation or ion-exchange chromatography to purify the phage particles. The third protocol describes a rapid procedure for isolating phage DNA, suitable for small-scale liquid lysates.

PREPARING DNA BY STEP- AND EQUILIBRIUM-GRADIENT CENTRIFUGATION

BASIC PROTOCOL

A scaled-up liquid lysate is used to prepare a large quantity of highly purified phage DNA. Phage is separated from cellular debris by a CsCl step gradient followed by equilibrium-gradient centrifugation. Two alternate sets of steps are provided for extracting λ DNA from CsCl-purified phage particles. In the first method, CsCl is removed by dialysis and the DNA is extracted by phenol and chloroform. In the second approach, phage DNA is extracted directly from CsCl-purified phage particles with high-grade formamide.

Materials

5× polyethylene glycol (PEG) solution
Suspension medium (SM; *UNIT 1.11*)
Potassium chloride
CsCl solutions
Low-salt buffer
Buffered phenol (*UNIT 2.1*)
Chloroform
2 M Tris·Cl (pH 8.5)/0.2 M EDTA (optional, for formamide extraction)
Formamide (*very* high grade, preferably recrystallized; optional)
TE buffer, pH 8.0 (*APPENDIX 2*)
Beckman JA-10, JA-20, SW-28, and VTi50 rotors and bottles/tubes (or equivalents)
3-ml syringe with 25-G needle
Beckman VTi50 quick-seal tubes
Additional materials for preparing liquid phage lysate (*UNIT 1.12*), titering lambda phage (*UNIT 1.11*), and quantitation of DNA (*APPENDIX 3*)

Prepare and concentrate the phage

1. Use 25 ml liquid lysate to make a 1000-ml lysate (see *UNIT 1.12*).
2. Split the lysate into two JA-10 centrifuge bottles and spin 10 min at 10,000 rpm (17,700 × *g*), 4°C, to remove cell debris.
3. Transfer supernatant to a 1000-ml graduated cylinder and add 5× PEG solution to a final concentration of 1×. Invert gently to mix. Let sit overnight at 4°C.

   The PEG solution causes the phage to precipitate.

4. Remove ~50 ml of supernatant and save for step 5. Pour off remaining supernatant, being careful not to lose any of the white precipitate.
5. Transfer precipitate to Nalgene centrifuge tube. Rinse cylinder with saved supernatant and transfer to centrifuge tube. Spin 10 min in JA-20 rotor at 5000 rpm (3000 × *g*), 4°C.

6. Place centrifuge tubes on ice. Remove the top layer, being careful not to remove any of the thick white phase, which contains the PEG solution and the phage.

7. Resuspend the white phase in a minimum volume of SM. Transfer to a 125-ml flask.

   The volume of the suspension medium added should not be more than three times the volume of the white phase.

8. Determine the amount of solid KCl needed to make a 1 M solution. Add this amount of KCl in four aliquots of approximately equal size, mixing well after each addition. Let sit on ice for 15 to 30 min.

   Adding KCl precipitates the PEG solution slowly while leaving the phage behind.

9. Transfer to Nalgene centrifuge tube and spin 10 min in a JA-20 rotor at 10,000 rpm (12,100 × *g*), 4°C.

   The PEG solution will be pelleted and the phage will remain in the supernatant.

10. Measure the phage titer and keep the supernatant in a 16- or 18-mm glass test tube.

    The phage titer should be ~1 × 10^{12} to 5 × 10^{13} pfu/ml.

Isolate the phage particles

11. Pour a CsCl step gradient in an SW-28 centrifuge tube as follows:

    First layer: 3.5 ml CsCl solution, d = 1.7 g/ml
    Second layer: 2.5 ml CsCl solution, d = 1.5 g/ml
    Third layer: 2.5 ml CsCl solution, d = 1.3 g/ml

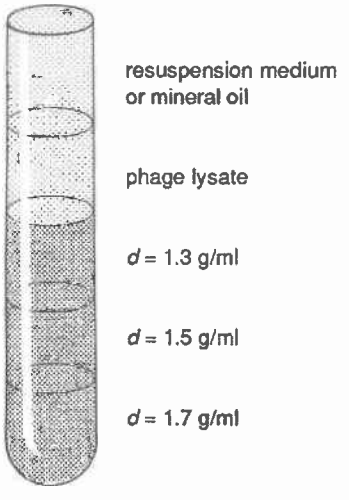
    The layers must be added very slowly to avoid mixing.

12. Carefully layer the lambda lysate (supernatant from step 10) on top of this gradient (see sketch 1.13A).

13. Fill tube to just below the top with SM. Spin 2 hr in an SW-28 rotor at 24,000 rpm (104,000 × *g*), 4°C.

14. Recover the phage band by inserting a 3-ml syringe with a 25-G needle into the side of the tube just below the band and drawing it into the syringe (see sketch 1.7A in CsCl/EtBr plasmid prep, *UNIT 1.7*).

    Usually, three bands are visible: one blue phage band at the lowest gradient, one blue



CsCl step gradient

Sketch 1.13A

band containing empty phage heads, and one white cell debris band. Occasionally one or two of these bands are missing. This is not a problem as long as the band that is visible is in the d = 1.5 g/ml layer. If no band is visible, there are probably not enough phage to make it worthwhile continuing the procedure. If the layers are not clearly defined, but a bluish band appears at about the right place, recover the band and determine its density by weighing 100 µl of the lysate. If its density is ~1.5, it probably is phage.

15. Transfer the phage to Beckman VTi50 quick-seal tubes. Fill tubes with CsCl solution (d = 1.5 g/ml).

    About half the weight of the phage particle is protein; the other half is DNA. Phage DNA is denser than phage proteins. Thus, phages with genomes larger than wild-type are denser than phages with wild-type-length genomes, and phages with smaller genomes are less dense.

16. Spin 24 hr in VTi50 rotor at 30,000 rpm (81,500 × g), 4°C.

17. Remove the band as shown in sketch 1.7A of CsCl/EtBr plasmid prep (*UNIT 1.7*), using a 3-ml syringe and a 25-G needle. Only one band should be visible.

Extract the phage DNA

Two methods are presented below for extracting DNA from purified phage. In steps 18a to 21a, cesium chloride is removed by dialysis prior to extraction of DNA with phenol and chloroform. In steps 18b to 21b, DNA is extracted directly from the isolated phage with high-grade formamide. The latter approach is quicker and possibly gentler.

Dialysis and phenol/chloroform extraction:

18a. Dialyze, with stirring, in 500 ml low-salt buffer for ≥4 hr at 4°C. Repeat twice.

19a. Extract three times by agitating gently for 20 min with an equal volume of buffered phenol.

20a. Extract twice using an equal volume of chloroform.

21a. Dialyze, with stirring, in 500 ml TE buffer, pH 8.0, for 8 hr at 4°C. Change buffer once. Proceed to step 22.

Residual phenol and chloroform are removed by dialysis rather than ethanol precipitation because purified phage DNA is very difficult to resuspend.

Formamide extraction:

18b. Measure the volume of phage band from step 17. Add 0.1 vol of 2 M Tris·Cl (pH 8.5)/0.2 M EDTA and invert to mix.

19b. Add 1 vol formamide, mix, and let stand 30 min at room temperature.

20b. Add 2 vol (each equal to the original volume of phage band in step 18b) of 100% ethanol at room temperature. Mix gently and microcentrifuge 1 to 2 min.

21b. Discard supernatant and rinse pellet with 70% ethanol. Remove all droplets of ethanol with a drawn-out pipet and dissolve the moist pellet in TE buffer, pH 8.0.

22. Measure DNA concentration as described in *APPENDIX 3*.

ALTERNATE PROTOCOL

PREPARING DNA USING DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

This protocol employs an ion-exchange resin to preferentially bind contaminants in crude phage lysate (*E. coli* DNA, RNA, and protein) while phage particles pass through the column. The result is a highly purified phage preparation. The DNA is then extracted from the purified phage with organic solvents and precipitated with ethanol. The method is simple, rapid, and does not require DNase, RNase, or CsCl density gradient centrifugation of either phage particles or DNA. The high-quality λ DNA obtained is suitable for cloning, sequencing, restriction enzyme digestion, ligation, and in vitro packaging.

Materials

TM buffer
2% sodium dodecyl sulfate (SDS; optional)
0.1 M EDTA (optional)
Sodium chloride
Polyethylene glycol (PEG) 8000
DEAE-cellulose (microgranular anion exchanger; Whatman DE52 #4057-050)
0.05 N HCl
10 M NaOH
Sodium azide
5 M NaCl
Ice-cold 100% isopropanol
TE buffer, pH 8.0 (*APPENDIX 2*)
25:24:1 phenol/chloroform/isoamyl alcohol (*UNIT 2.1*)
3 M sodium acetate, pH 6.0
70% ethanol and ice-cold 100% ethanol
Beckman JA-14 and JA-20 rotors (or equivalents)
15- and 30-ml Corex centrifuge tubes
10-ml disposable syringe (1.4 cm-i.d., optional; Becton Dickinson) *or* 1.5 × 10–cm standard glass or disposable column (Bio-Rad)
Glass-fiber filter or glass wool (optional)
Additional reagents and equipment for preparing liquid or plate lysate (*UNIT 1.12*), titering lambda phage (*UNIT 1.11*), agarose gel electrophoresis (*UNIT 2.5*), and phenol extraction/ethanol precipitation (*UNIT 2.1*)

Prepare concentrated crude phage lysate

1. Prepare liquid or plate lysate and determine the phage titer. The lysate should contain 1-2 × 10^{10} pfu/ml in a volume of 200 ml.

   When using plate lysates, collect phage in TM buffer (instead of SM) from six big (150 mm) or fifteen small (90 mm) petri dishes and adjust volume to 200 ml.

   Alternatively, a 0.7% agarose minigel can be used to detect λ DNA in phage lysates. Treat 20 μl lysate with 2 μl of 2% SDS and 2 μl of 0.1 M EDTA for 5 min at room temperature. Load onto gel. Include size standard and control (nontreated) lanes. In this gel, the SDS/EDTA-treated lysate sample should show a distinct λ DNA band compared to the nontreated sample (Fig. 1.13.1).

2. Add 5.8 g NaCl (0.5 M final) and 20 g PEG 8000 (10% wt/vol final) to 200 ml phage lysate.

   If the phage titer is low, it may be necessary to add NaCl first to release phage adhering to the debris. Remove debris by centrifuging 10 min in JA-14 at 6000 rpm (5500 × g), 4°C, and then add PEG. Dissolve the PEG flakes by gentle stirring and place on wet ice 1 hr. This is enough time to precipitate most of the phage.

3. Separate precipitated phage by centrifuging 10 min in a JA-14 rotor at 6000 rpm, 4°C.

   Drain liquid from the bottles by placing in an inverted position; and shake by hand to remove any remaining liquid.

4. Resuspend phage pellet in 3 ml TM buffer and transfer to a 15-ml Corex centrifuge tube. Add 3 ml chloroform, mix gently, and centrifuge 10 min in a JA-20 rotor at 5000 rpm (3000 × *g*), 4°C. Collect the phage-containing upper aqueous phase without disturbing the PEG interface.

   Corex tubes make it easy to see the PEG interface and the well-separated supernatant.

5. Add 3 ml TM buffer to the tube, mix, and centrifuge as in step 4. Save the aqueous fraction and combine with aqueous fraction from step 4. Adjust the volume to 6 ml with TM buffer.

   This step removes phage particles trapped at the PEG interface.

Prepare the DEAE-cellulose column

6. Prepare a slurry of DEAE-cellulose by adding several volumes of 0.05 N HCl. Make sure the pH of the solution is below 4.5.

   It is convenient to prepare enough DEAE-cellulose for 30 to 40 columns. Each column requires ~9 ml resin (for 200 ml phage lysate).

7. While stirring, add 10 M NaOH until the pH approaches 7.5.

8. Let the resin settle, decant or aspirate, and equilibrate the resin with TM buffer (see manufacturer's instructions).

   Make sure the DEAE-cellulose is completely equilibrated with TM buffer. This may require repeating step 8 four or five times. This process also removes fine particles from the resin.

9. Adjust the slurry to 75% resin and 25% TM buffer.

   For long-term storage (several months), add NaN_3 to a final concentration of 0.02% and place at 4°C.

10. Pour 9 ml resin in a 10-ml disposable syringe (1.4-cm i.d.) or a 1.5 × 10–cm column. This will result in a bed height of 5 to 6 cm, appropriate for 200 ml of phage lysate.

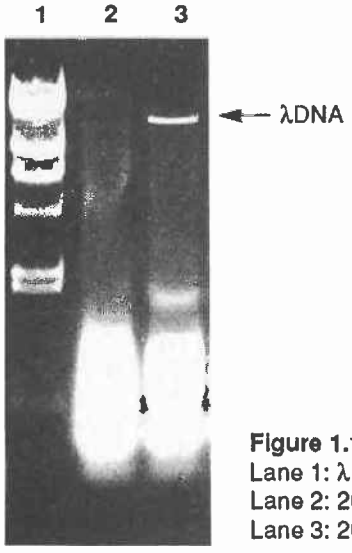


Figure 1.13.1 Agarose minigel of bacteriophage lambda lysates.
Lane 1: λ DNA digested with *HIN*dIII.
Lane 2: 20 µl of lysate without SDS and EDTA.
Lane 3: 20 µl of lysate treated with 2% SDS and 0.1 M EDTA.

When using a disposable syringe, place a glass-fiber filter or glass wool at the bottom of the syringe to support the resin. Attach a small piece of tubing with a stopcock to the syringe tip to control the flow. Column preparation is easier with a standard glass or disposable column. See UNIT 10.10 for complete discussion of ion-exchange chromatography and Figure 3.4.1 for a sketch of homemade column setup.

Isolate the DNA

11. Load 6 ml crude phage solution from step 5 onto DEAE-cellulose column and elute with 10 ml TM buffer. Discard the first 3 ml of the eluate (void volume); collect the next 13 ml in a 30-ml Corex tube.

    The 13-ml eluate may be collected in 1-ml fractions, if desired. Generally, phage elute immediately after the void volume. Fractions containing high-titer phage appear bluish due to light scattering. This indicates that the column is working well. Phage purification can also be confirmed by analyzing 5 µl of each fraction on an agarose minigel (Fig. 1.13.1).

12. Add 2 ml of 5 M NaCl (400 mM final) and 10 ml of ice-cold 100% isopropanol (40% final) to the 13-ml eluate. Place at –20°C for 15 min.

13. Centrifuge 10 min in JA-14 rotor at 5000 rpm (3800 × *g*), 4°C, and discard supernatant.

    Invert centrifuge tubes until liquid is completely drained. Using tissue paper, wipe the walls of the centrifuge tube without touching the phage pellet. The pellet will appear as a feathery design adhering to wall of the tube. If there is not a sufficient pellet, abandon the experiment and start again from new lysate.

14. Resuspend the phage pellet in 0.8 ml TE buffer, pH 8.0, and divide equally into two 1.5-ml microcentrifuge tubes.

15. Extract once with phenol, twice with phenol/chloroform/isoamylalcohol, and finally with chloroform. Repeat extractions until no visible protein precipitate is seen at the interface.

16. Precipitate λ DNA by adding 1/10 vol of 3 M sodium acetate, pH 6.0, and 2 vol ice-cold 100% ethanol.

    In general, λ DNA forms a fibrous precipitate immediately after the addition of ice-cold ethanol at room temperature. A white cloudy precipitate indicates leftover protein. The λ DNA may be spooled out with a glass rod or condensed into a globule by gentle mixing.

17. Rinse the DNA pellet with 70% ethanol, air dry, and resuspend in TE buffer, pH 8.0. After dissolving completely, measure DNA concentration (*UNIT 1.7*) and adjust to 200 µg/ml in TE buffer. Store at 4°C.

    It may take several hours for the λ DNA to go into solution.

PREPARING DNA FROM SMALL-SCALE LIQUID LYSATES

ALTERNATE PROTOCOL

This protocol is useful for making small quantities of DNA to be used for restriction analysis. Phage are concentrated by centrifugation and their capsids are destroyed with phenol. The DNA is then ethanol precipitated.

Additional Materials

5 mg/ml DNase (*UNIT 3.12*)
10 mg/ml DNase-free RNase (*UNIT 3.13*)
0.05 M Tris·Cl, pH 8.0
3 M sodium acetate, to pH 4.8 with acetic acid

1. To approximately 50 ml liquid phage lysate (*UNIT 1.12*), add 10 µl of 5 mg/ml DNase and 25 µl of 10 mg/ml DNase-free RNase. Incubate 1 hr at 37°C.

   This treatment will degrade the bacterial DNA and RNA released during lysis. The viscosity of the mixture should decrease.

2. Centrifuge 1½ hr at 27,000 rpm in an SW-28 rotor (132,000 × *g*), 4°C.

   Alternatively, pellet the phage by spinning 2¼ hr in JA-20 rotor at 20,000 rpm (48,000 × g), 4°C. The pellet obtained in this manner will resuspend somewhat more easily.

3. Discard supernatant. Invert the tubes on an absorbent surface, e.g., paper towels, to remove any remaining liquid. Resuspend the phage pellet in 200 µl of 0.05 M Tris·Cl, pH 8.0.

   A small translucent pellet should be visible after the tubes are inverted.

4. Transfer the solution to a microcentrifuge tube and add 200 µl buffered phenol. Vortex 20 min or shake 20 min in microcentrifuge tube shaker. Spin 2 min in microcentrifuge and save the aqueous (top) layer. Repeat phenol extraction.

   Phenol denatures the phage capsids and releases the DNA. This denatured capsid protein appears as a thick white precipitate at the phenol/water interface. Vigorous agitation is necessary because the pellet is difficult to resuspend.

   There should be less white precipitate after the second phenol extraction. If there is still a large amount at the interface, do a third extraction.

5. Add 200 µl chloroform, shake well, and spin in microcentrifuge briefly. Save the aqueous (top) layer. Repeat.

6. Add 20 µl of 3 M sodium acetate, pH 4.8, and precipitate DNA with 2 vol of 100% ethanol at room temperature. Spin in microcentrifuge for 10 min.

7. Remove supernatant. Wash pellet by adding 1 ml of 70% ethanol and spinning 5 min.

8. Remove supernatant. Dry pellet under a vacuum and resuspend the DNA in 100 µl TE buffer, pH 8.0.

   The DNA will resuspend more quickly if the pellet is still slightly wet.

   3 µl of the DNA suspension should be used for a restriction digest.

REAGENTS AND SOLUTIONS

CsCl solutions

d = 1.3 g/ml: 31.24 g CsCl + 68.76 ml H_2O
d = 1.5 g/ml: 45.41 g CsCl + 54.59 ml H_2O
d = 1.7 g/ml: 56.24 g CsCl + 43.76 ml H_2O

The equation for preparing the CsCl solutions is: % w/w = 137.48 − 138.11/d. This calculation assumes the CsCl has no water in it. Typically, CsCl from the shelf will have adsorbed water from the air, and the densities of these stock solutions will be lower than claimed. However, in our experience, the phage band is always found in the middle layer of the step gradient.

Low-salt buffer

0.05 M NaCl
0.05 M Tris·Cl, pH 7.5
0.01 M $MgSO_4$

5× polyethylene glycol (PEG) solution, 600 ml

207 g Carbowax (PEG 6000)
6 g dextran sulfate
49.5 g NaCl
350 ml H_2O

TM buffer

50 mM Tris·Cl, pH 7.5
10 mM $MgSO_4$

COMMENTARY

Background Information

This unit provides three methods for extracting and purifying DNA from intact phage particles. The three methods differ in the amount of starting material or phage lysate, and in the manner in which phage are concentrated and purified before lysing and DNA release.

The large-scale preparation (basic protocol) is useful when large quantities of highly purified DNA (over 200 µg) are required—for example, in the construction of libraries. It is based on traditional methods of bacteriophage lambda isolation, involving precipitation of phage from the lysate by polyethylene glycol (PEG) and subsequent purification by CsCl step and equilibrium density-gradient centrifugation (Yamamoto et al., 1970; Davis et al., 1980; Maniatis et al., 1982). It is necessary to use two different gradients to purify phage away from the considerable amount of cellular debris present in a large lysate. Following phage purification, DNA is extracted from capsids by one of two approaches. In one method, phage proteins are removed by a series of phenol extractions and resulting phage DNA is dialyzed in TE buffer to remove any remaining phenol or chloroform. In the other method, DNA is extracted directly from the capsids with high-grade formamide.

The first alternate protocol describes a moderate-scale procedure, capable of yielding enough pure lambda DNA (about 200 µg) for most standard DNA manipulations, such as cloning, sequencing, in vitro packaging, and Southern blotting. Phage particles are separated from cellular components in the lysate by ion-exchange chromatography, resulting in a high degree of purification in a single step. The chromatographic purification works as follows. DEAE (diethyl aminoethyl)-cellulose is an anion-exchange resin—i.e., it has positively charged groups that adsorb negatively charged molecules in buffer of near neutral pH and medium ionic strength. The major cellular contaminants in bacteriophage lysates are negatively charged (polyanionic) molecules (DNA, RNA, and proteins) which are preferentially adsorbed onto the positively charged groups of DEAE-cellulose (Creaser and Taussig, 1957; Benson and Taylor, 1984; Shuang-Young, 1986).

Two different ways to use column chromatography with Whatman DE52 anion exchange resin for phage purification have been reported. In the procedure described here, cellular contaminants are bound to the column, leaving phage particles free (Reddy et al., 1988;

White and Rosenzweig, 1989). Because SDS and proteinase K are not used in the nucleic acid extraction, the resulting DNA can be used with restriction endonucleases and DNA-modifying enzymes without further purification, and has been used successfully to make genomic expression libraries (Webb et al., 1989). Alternatively, binding phage particles to the resin with subsequent elution using a high-salt buffer (Helms et al., 1985) is used for processing several clones simultaneously to obtain a few micrograms of DNA for subcloning.

The second alternate protocol describes a rapid, small-scale method for phage purification which yields small amounts of moderately pure DNA, suitable for restriction analysis. In this procedure, phage are pelleted out of solution with a high-speed spin, and host nucleic acids are destroyed using RNase and DNase. (These enzymes have no effect on phage DNA as it is still packaged in phage heads.) Phage DNA is subsequently extracted from the capsids with phenol and is concentrated with ethanol precipitation.

Critical Parameters

Regardless of procedure, most failures to isolate lambda DNA are due to low phage titer in the initial lysate. Thus, hours of wasted time can be spared by first titering the phage lysate. Phage can be titered by dilution plating (*UNIT 1.11*); if the titer is less than 10^9 pfu/ml, a new lysate should be made. An alternative and usually superior method to determine the amount of DNA is to treat 20 µl of lysate with SDS/EDTA followed by electrophoresis on an agarose minigel (*UNIT 2.5*). The gel assay takes less time and provides a more accurate estimation of DNA present in both viable and nonviable phage particles. Figure 1.13.1 (lane 3) shows a typical gel resolving relative amounts of cellular nucleic acids and λ DNA (~50 ng) present in a lysate. One should be able to visualize ≥10 ng of λ DNA in the band. If there is no visible band, it is advisable to start from fresh lysate.

In chromatographic purification, the ratio of lysate to column size is an important variable. A 9-ml bed volume is sufficient for 200 ml of liquid lysate (at 10^{10} pfu/ml); if more lysate is used (or, if lysate has more highly concentrated cellular debris), the bed volume should be increased proportionately. If the column is overloaded, cellular DNA and RNA may coelute with the phage (to assess this, monitor column fractions with sample of crude lysate by running on an agarose gel). If DNA is contaminated, column fractions can be pooled and treated with DNase and RNase (*UNITS 3.12 & 3.13*) before organic solvent extraction of phage DNA. One should carefully prepare, equilibrate, and pack DEAE-cellulose according to instructions in the Whatman information leaflet (see also *UNIT 10.10*). Note that the type of DEAE-cellulose (Whatman DE52), as well as the pH and ionic strength of the buffer (use TM buffer recipe provided), are important. Chloroform-lysed cultures often contain very viscous, high-molecular-weight chromosomal DNA which can block the DEAE column. Such a lysate can be treated with DNase before loading onto the column.

After organic extraction, it may be preferable to dialyze rather than precipitate phage DNA, because large DNA can be difficult to resuspend. Lambda DNA is large and easily sheared after it is isolated. When intact DNA is desired (e.g., when phage arms are to be prepared for making a library), the DNA should be treated with special care: shaken, swirled, or vortexed very gently to mix.

Anticipated Results

Yield of λ DNA from each of these procedures depends upon the phage titer and the original lysate volume. If the initial lysate titer is around $1\text{-}2 \times 10^{10}$ pfu/ml, then 1 ml of phage lysate ought to yield ~1 µg of DNA; that is, 2×10^{10} phage particles gives ~1 µg purified phage DNA. Thus, the large-scale procedure, starting with 1000 ml of lysate, gives ~1 mg of phage DNA; the DEAE-column procedure (200 ml lysate) results in ~200 µg DNA; and the small-scale procedure (50 ml lysate), provides ~50 µg DNA.

Time Considerations

The large-scale prep employing gradient purification typically takes at least 3 days: ~30 min the first day to set up the PEG precipitation; ~5 hr the next day to spin down and collect the precipitate, run the step gradient, and load the equilibrium gradient; and ~30 min the third day to collect and phenol extract the phage band, followed by two dialysis steps lasting 8 hr. The phage lysates made as intermediates can be stored for months at 4°C without decreasing in titer. The small-scale prep takes ~4 to 6 hr and it is fairly easy to process a number of samples at once.

Column-purified phage and DNA isolation can be completed in 4 hr, starting from liquid

or plate lysate. Preparation of the crude phage solution for the column takes ~2 hr. Another 2 hr are needed for column setup, column loading, elution, and DNA extraction. It is usually most convenient to process four to five different columns simultaneously. The DEAE-cellulose slurry can be prepared in advance and stored at 4°C for several months.

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White, B.A. and Rosenzweig, S. 1989. A reliable method for the purification of bacteriophage λ DNA. *Biotechniques* 7:694-695.

Key References

PEG precipitation

Yamamoto, K.R., Alberts, B.M., Benzinger, R., Lawhorne, L., and Treiber, G. 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* 40:734-744.

Step gradient and equilibrium gradient

Davis, R.W., Botstein, D., and Roth, J.R. 1980. A Manual for Genetic Engineering: Advanced Bacterial Genetics, pp. 70-113. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Includes a number of other protocols for preparation of DNA from lambda.

Ion-exchange purification

Reddy et al. 1988. See above.

Describes the details of the DEAE-cellulose column chromatography of λ DNA upon which this protocol is based. Also describes a large-scale isolation procedure of λ DNA from a 1-liter liquid lysate using a 45-ml bed volume DEAE column.

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VECTORS DERIVED FROM FILAMENTOUS PHAGES

Introduction to Vectors Derived from Filamentous Phages

Many vectors in current use are derived from filamentous phages. These vectors are used because DNA inserted into them can be recovered in two forms—double-stranded circles and single-stranded circles. Foreign DNA is inserted into double-stranded vector DNA and, then, reintroduced into cells by transformation. Once inside the cells, double-stranded DNA replicates, giving rise both to new double-stranded circles and to single-stranded circles derived from one of the two strands of the vector. Single-stranded circles are packaged into phage particles and secreted from cells (which do not lyse). Centrifugation of a culture of infected cells yields a supernatant that is full of particles containing only a single strand of the phage DNA. This ready availability of single-stranded DNA has made possible new procedures for sequencing DNA (Chapter 7), mutagenesis (Chapter 8), and other techniques described in this book.

Techniques to isolate double-stranded DNA using these vectors are described in *UNIT 1.15*.

DEVELOPMENT AND USE OF FILAMENTOUS PHAGE VECTORS

Prototypes of the filamentous phage vectors are the M13mp derivatives (see Figs. 1.14.1 and 1.14.2). These vectors were developed by Joachim Messing and his co-workers, who also developed and disseminated simple and powerful techniques for working with them. The M13mp vectors are viable phages. Foreign DNA is inserted into a polylinker (a stretch of DNA that contains contiguous restriction sites) located in an inessential region

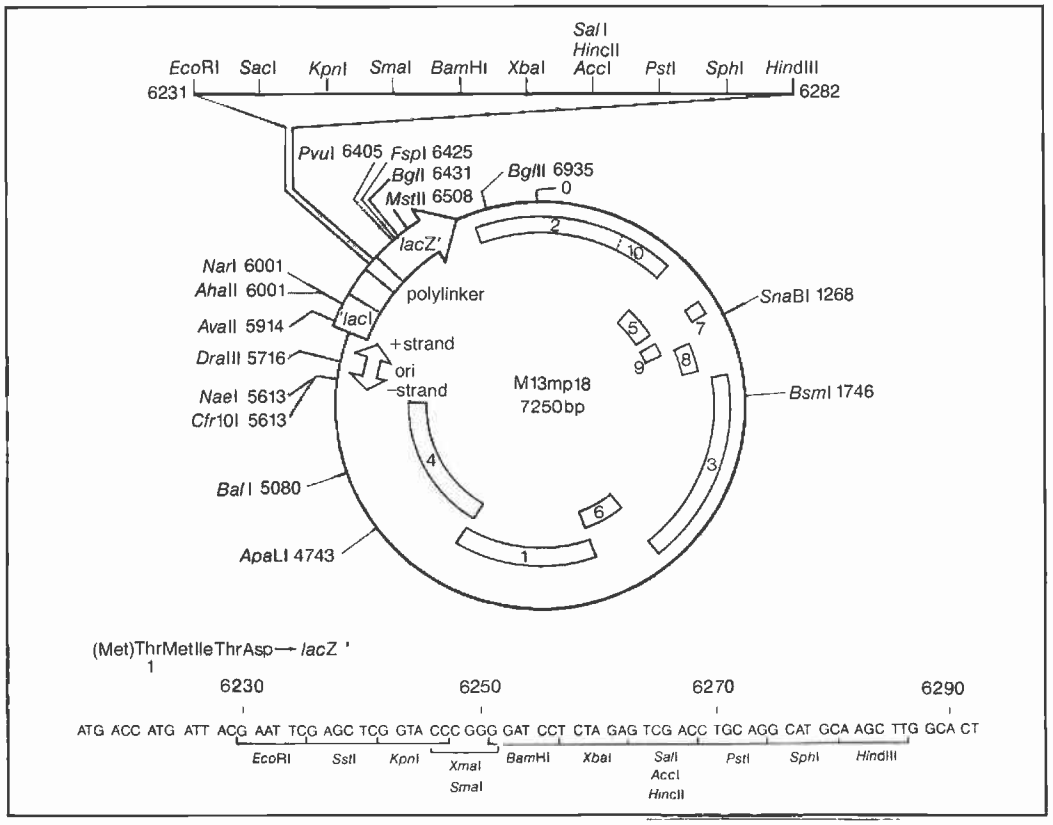

Figure 1.14.1 M13mp18. M13mp18 is one of the M13mp vectors made by Messing and colleagues. Insertion of DNA into the polylinker inactivates the *lacZ* alpha fragment. When insert-containing phages are plated under appropriate conditions (*UNIT 1.15*), they form colorless plaques; vectors that do not contain inserts form blue plaques (Yanisch-Perron et al., 1985, and references therein).

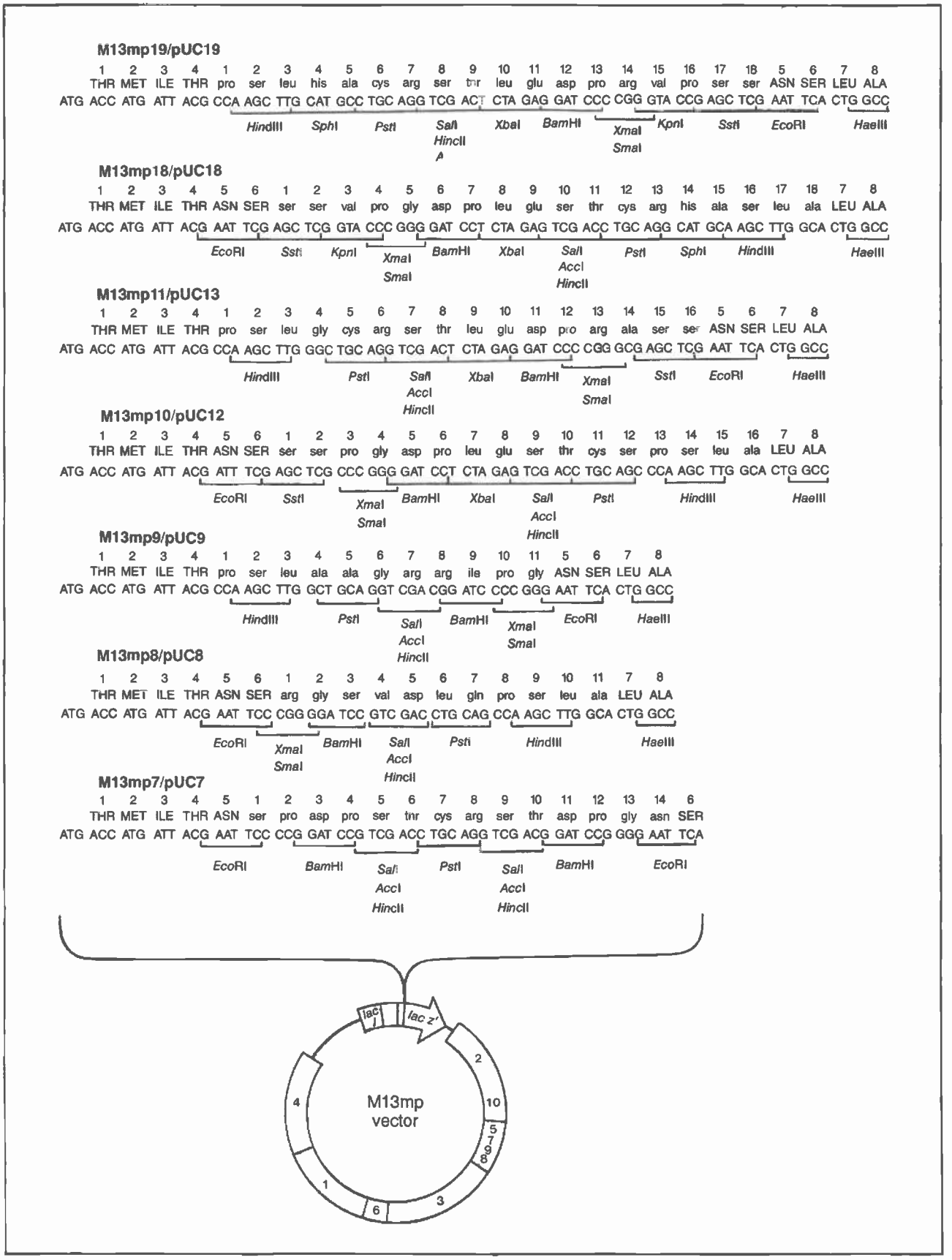

Figure 1.14.2 M13mp/pUC polylinkers. Sequence of polylinkers in the commonly used members of these two series of vectors (Yanisch-Perron et al., 1985, and references therein). Amino acids that have been added to the *lacZ* gene product by insertion of the polylinker are shown in lower case letters. The bracket shows location of polylinkers on vector.

of the phage genome. The polylinker is embedded in-frame within an alpha fragment of the *lacZ* gene. M13mp derivatives form blue plaques on lawns of cells that contain the *lacZ* omega fragment on plates with Xgal and IPTG. Double-stranded DNA is purified from phage-infected cells, cut within the polylinker with restriction enzymes, and foreign DNA is ligated to the cut vector. Insert-bearing phages form white plaques. Infected cells from the center of white plaques are grown in liquid culture then centrifuged to yield supernatants full of phage particles containing a single strand of DNA. Phage particles are concentrated and pure single-stranded DNA is extracted from them and used in other procedures.

Two inadequacies of the M13mp phages sparked development of subsequent generations of vectors. First, it is not always easy to obtain large amounts of double-stranded DNA. This is because DNA is obtained by lysing infected cells, but infected cells grow more slowly than uninfected cells, and there is no easy selection for cells that retain the phage. Second, phages that contain insertions of more than a few hundred nucleotides sometimes give rise to progeny phage in which some of the inserted DNA was deleted. This may be due to the fact that cells infected with large phages grow even more slowly than cells infected with small phages, so that cells containing deletion derivatives of phages with large inserts have a growth advantage.

In order to overcome these disadvantages, many vectors were developed (e.g., pEMBL) that contain a short stretch of DNA that includes the phage origin of replication. These vectors also contain a pMB1-derived origin of replication, a β-lactamase (ampicillin-resistance) gene, and a polylinker embedded within an alpha-complementing fragment of *lacZ*. The vectors, which are much smaller than the M13mp phages, are introduced into the cell by transformation; their continued presence can be ensured by growth of the cells in ampicillin-containing medium. Since these vectors do not encode phage proteins required for DNA replication, vector DNA replicates inside the cells using the pMB1 origin of replication. However, when cells containing these vectors are superinfected with wild-type *helper phage*, the phage origin of replication becomes active and single-stranded progeny phages are secreted into the medium together with progeny helper phage. Contamination with helper phage usually does not interfere with most applications because the helper does not contain genes encoding *lacZ* or ampicillin resistance, nor is it likely to contain any piece of nucleic acid used as a hybridization probe. Vectors developed even more recently (e.g., pUC118, pBluescribe) are designed to be used with a slightly improved helper phage and also often include SP6 or T7 phage polymerase promoters reading into the polylinker (see Fig. 1.5.3 and *UNIT 1.15* for details).

LIFECYCLE OF FILAMENTOUS PHAGES

The filamentous phages (f1, M13, and fd) are single-stranded DNA phages that are male-specific—that is, they infect male *E. coli* strains that contain an F factor (F^+, F', or Hfr; see *UNIT 1.4*). These three phages are essentially independent isolates of the same phage, and differ only by a few nucleotide substitutions. The phage particle is shaped like a long thin tube. Its coat is composed primarily of thousands of monomers of the gene 8 product. The genome consists of a single-stranded, circular DNA molecule, 6407 nucleotides long, which runs down the length of the tube. In addition to coding for ten proteins, the genome contains an intergenic space (IG) between gene 4 and gene 2. The IG contains origins for (+)-and (−)-strand DNA synthesis, a signal for packaging the (+) strands into phage particles, and a transcription terminator. (A genetic map of the phage is shown in Figure 1.14.3.) Filamentous phages are useful cloning vectors because there appears to be no size limit for packaging; if longer genomes are generated, they are packaged into longer phage particles. The phage particle contains ~2700 monomers of the major coat protein, the product of gene 8, and several minor coat proteins at the ends. One of the minor coat proteins, the product of gene 3, attaches to the receptor at the tip of the F pilus of the host *E. coli*. Upon binding, the pilus is thought to retract, bringing the phage in contact with the bacterial cell surface. The coat proteins are removed from the phage particle and inserted into the bacterial cell membrane. After uncoating, the infecting circular single-stranded DNA is brought into the cytoplasm in a process that remains unclear.

Phage DNA replication takes place in three stages within the infected cell. In stage one (complementary strand synthesis), a complementary (−) strand of phage DNA is synthesized. This synthesis converts the infecting single-stranded circular DNA, the (+) strand, into double-stranded replicative-form (RF) DNA. Synthesis of the (−) strand begins at the (−)-strand origin (within the IG), and is carried

out by the host *E. coli* replicative enzymes.

In the second stage of replication, the intracellular pool of RF DNA is increased by the sequential action of both replication origins. Rolling-circle type replication from the (+)-strand origin is followed by conversion of the progeny single-stranded circles to double strands by replication starting at the (–)-strand origin. The resulting RF DNA molecules are intermediates in DNA synthesis and transcription templates for the synthesis of the phage-encoded proteins. Transcription proceeds in the same direction as (+)-strand rolling-circle replication. It is suspected that transcription into the replication fork, in the direction of the (–) strand, would inhibit replication, as proposed for the replication of the *E. coli* chromosome (Brewer, 1988). Although this idea has not been rigorously tested, it is attractive because it provides an explanation for two common cloning problems: why certain pieces of DNA are not clonable, and why, occasionally, only one of two possible orientations of an insert are sometimes recovered in a subcloning experiment.

The (+)-strand replication origin that directs rolling-circle replication has a bipartite structure. It consists of an essential core origin region (~50 bp) and an adjacent A+T–rich "enhancer" sequence (~100 bp) which increases replication ~100-fold. The core origin binds the initiator protein (gene 2 protein) and the enhancer binds the *E. coli* integration host factor (IHF). The polylinker cloning site in cloning vectors of the M13mp series disrupts the replication enhancer sequence, but these vectors have acquired compensatory mutations in gene 2 that restore efficient replication. Consequently, virtually any sequence can be introduced into the polylinker without disrupting DNA replication.

The gene 2 protein is a multifunctional protein that plays several roles in phage DNA replication. It binds cooperatively to the (+)-strand origin in two steps, bends the origin DNA, and introduces a specific nick in the (+) strand of RF DNA. The 3′-hydroxyl end of the nick serves as the primer for (+)-strand rolling-circle replication. (This activity makes the gene 2 protein a useful enzyme for producing uniquely nicked DNA molecules in vitro.) After nicking, the DNA molecules still need the gene 2 protein for unwinding and replication. Finally, upon completion of a round of synthesis, gene 2 protein cleaves and circularizes the displaced single strand (see *UNIT 1.15*).

The third stage of replication (single-strand production) occurs late in infection. This stage of DNA replication is asymmetric because the (–)-strand origin functions at a reduced level late in the infection and therefore mainly (+) strands are produced. These single-stranded circles, (+) strands, are packaged into phage

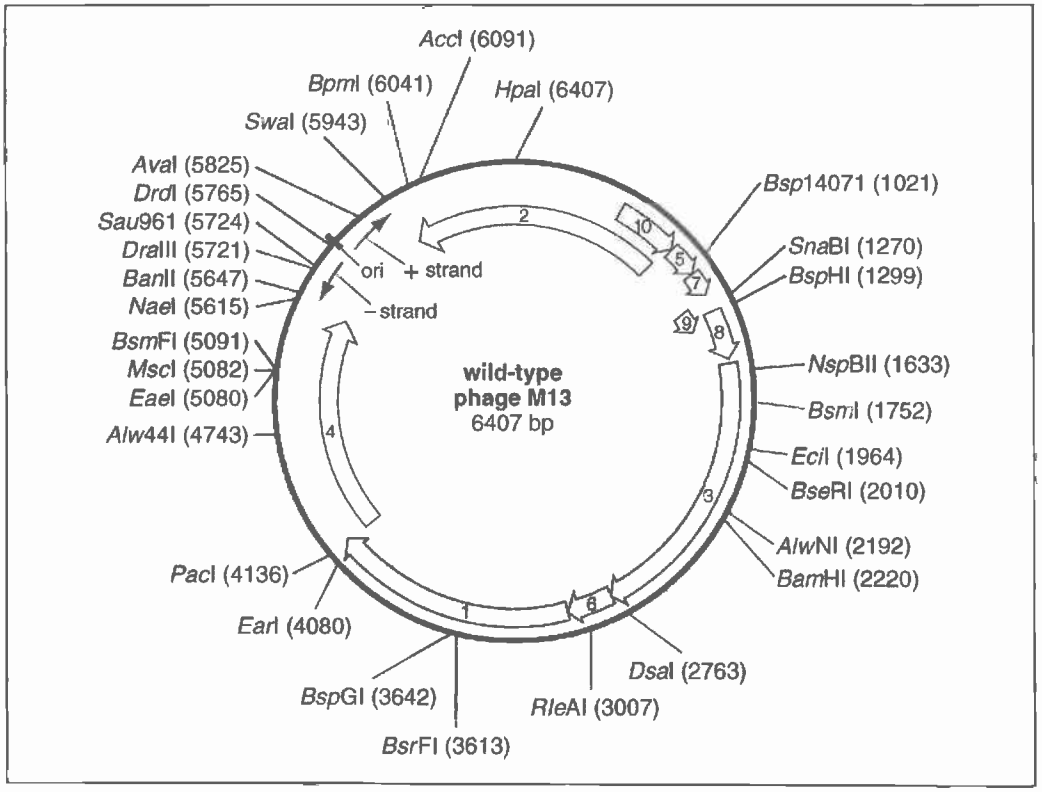

Figure 1.14.3 Lifecycle of wild-type M13, described in this unit and in Rasched and Oberer (1986).

particles for export instead of being converted into RF DNA.

Ordinarily, infection with filamentous phages is not lethal and the host cells do not lyse, although their growth rate slows ~2-fold. The reduced growth rate of the host cells accounts for the turbid plaques that the filamentous phage form on a lawn of sensitive cells. Surprisingly, several of the phage proteins have been found to be lethal to *E. coli* when expressed from a plasmid with enhanced replication (high copy number). Evidently, DNA replication in the infected cell is tightly regulated to prevent this lethality. The mechanism whereby this regulation is achieved is not completely understood, but the phage-encoded single-stranded DNA binding protein (gene 5 protein) appears to be required for it. In the steady state of infection, the RF DNA is maintained as a plasmid with a copy number of ~20 to 40, and phage are continuously exported at a rate of 100 to 200/hr. The infected state is quite stable with ~1 cell in every 1000 cells becoming uninfected each generation.

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Preparing and Using M13-Derived Vectors

Cloning vectors derived from filamentous phage are extremely useful because they allow cloned DNA to be isolated as either single- or double-stranded DNA. This unit contains protocols for preparing both forms of DNA and for characterizing inserts in M13-derived vectors. A protocol is also presented for preparing single-stranded DNA from plasmids using superinfection with helper phage. This method is advantageous because it allows cloned DNA to be maintained in the form of a plasmid while permitting single-stranded DNA to be isolated for DNA sequencing.

BASIC PROTOCOL

ISOLATING SINGLE M13-DERIVED VECTORS

To ensure homogeneity of stocks of native or insert-containing vectors, it is necessary to start from a single infected cell, or from a single plaque. This is achieved by plating out serial dilutions of an original culture stock.

Materials

E. coli strain infected with M13-derived vector (e.g., JM101 infected with M13mp18)
2× TY medium (*UNIT 1.1*)
20 mg/ml IPTG in H_2O (stored in aliquots at –20°C)
20 mg/ml Xgal in dimethylformamide (stored in aliquots at –20°C)
45°C top agarose (*UNIT 1.1*)
H plates, prewarmed to 37°C (*UNIT 1.1*)
5-ml Falcon tubes with caps or equivalent plastic tubes
37°C incubator

NOTE: *All materials coming into contact with E. coli must be sterile.*

1. Make a series of 1:10 dilutions of the infected strain or a phage stock in 2× TY medium. Place 100 µl of each dilution into separate 5-ml Falcon tubes with caps. Label the tubes in order to keep better track of them.

   The vector is usually stored in an infected host at –80°C in 20% glycerol.

   The vector can also be stored as isolated DNA or as phage. DNA is introduced into cells by transfection (UNIT 1.11); phage, by infection (described in steps 2 to 5 below). If the vector contains a plasmid origin and a drug-resistance marker, vector-containing cells can be isolated as single colonies on antibiotic-containing medium. If the vector forms viable plaques, vector-containing cells can be isolated by streaking single colonies from the tiny patch of cells in the center of the plaques.

   The following steps assume the vector is an M13mp vector, which contains a stretch of DNA that encodes the alpha fragment of β-galactosidase. These vectors make blue plaques on cells containing the β-galactosidase omega fragment (UNIT 1.4). Insertion of DNA into the polylinker inactivates the gene encoding the alpha fragment and gives rise to vectors that have colorless plaques.

2. To each tube add the following:

   200 µl noninfected bacteria grown to saturation
   10 µl IPTG
   40 µl Xgal
   3 ml 45°C top agarose

   It is convenient to prepare a larger batch of the first three ingredients beforehand.

3. Mix by rapidly inverting the tubes twice and pour on individual H plates prewarmed to 37°C.

4. Let the top agarose harden 10 min at room temperature and transfer the plates to a 37°C incubator.

5. After overnight growth, save only the plates that contain less than ~100 plaques.

PREPARING SINGLE-STRANDED PHAGE DNA FROM M13-DERIVED VECTORS

BASIC PROTOCOL

Cells containing the vector and the insert are grown up in liquid medium. The cells are collected by centrifugation, and single-stranded DNA is prepared from the phage particles in the supernatant.

Materials

E. coli strain (male-type, e.g., JM105)
M13-derived vector
2× TY medium (*UNIT 1.1*)
PEG solution (*UNIT 1.7*)
TE buffer (*APPENDIX 2*)
Buffered phenol (*UNIT 2.1*)
3 M sodium acetate
100% ethanol and cold 70% ethanol
Sterile toothpicks
37°C shaking water bath
Additional reagents and equipment for agarose gel electrophoresis (*UNIT 2.5*)

1. Inoculate 50 ml of 2× TY medium with 0.5 ml of an overnight culture of *E. coli*. The overnight should be grown from a single colony on a minimal plate that does not contain proline. Dispense 2-ml aliquots in 10-ml culture tubes. Inoculate each tube with a colorless phage plaque picked with a sterile toothpick.

   Many male strains of E. coli used for propagation of single-stranded phage (e.g., JM101 or JM105; Table 1.4.5) carry a deletion that removes the proA and proB genes, but also contain an F′ plasmid that contains the proA⁺ and proB⁺ genes. Picking a colony from a plate that lacks proline thus ensures that the cells contain the F′ plasmid, and that the phage will be able to infect them.

2. Shake at 37°C for 5 to 8 hr.

3. Transfer 1.5 ml of the culture to a microcentrifuge tube. Centrifuge 5 min at room temperature.

4. Pour 1.25 ml of the supernatant into a fresh microcentrifuge tube.

   To store the strain, resuspend the bacterial pellet by vortexing, adjust to 30% glycerol, and freeze at –80°C.

5. Add 250 µl PEG solution. Mix and leave for 15 min at room temperature.

6. Centrifuge 5 min and discard the supernatant.

   The phage pellet should be visible at this stage.

7. Centrifuge again for 5 min. Carefully remove all remaining traces of PEG using a drawn-out Pasteur pipet.

8. Add to the viral pellet 200 µl TE buffer. Resuspend the pellet completely, then add 100 µl buffered phenol.

9. Vortex 1 min, let stand for 5 min, vortex again, and centrifuge 5 min.

10. Remove 175 µl from the upper aqueous phase; transfer to a new microcentrifuge tube.
11. Add 20 µl of 3 M sodium acetate and 400 µl of 100% ethanol.
12. Leave ≥1 hr at –20°C to precipitate the DNA, or 15 min in a dry ice/ethanol bath.
13. Centrifuge 5 min, pour off the supernatant, add 1 ml cold 70% ethanol, centrifuge again, discard the supernatant, and dry briefly in a vacuum desiccator.

    For some applications of the single-stranded DNA, such as oligonucleotide-directed mutagenesis (UNIT 8.1), it is advisable to use very clean DNA. For these applications, aspirate off the supernatants from this step using a drawn-out Pasteur pipet.

14. Dissolve the pellet in 25 µl TE buffer.

    Yield can be as high as 10 µg DNA/ml culture.

15. Analyze 1 to 2 µl of the DNA solution by electrophoresis on a 1% agarose gel and store the remainder at –20°C.

BASIC PROTOCOL

PREPARING DOUBLE-STRANDED REPLICATIVE-FORM DNA

The disadvantage of many protocols for isolating double-stranded replicative-form (RF) DNA is that the DNA is isolated late in the infectious cycle, at a time when it is least abundant. The following protocol involves isolation of double-stranded DNA from chloramphenicol-treated cells. Addition of chloramphenicol at a low concentration (15 µg/ml) soon after infection prevents the accumulation of the phage gene 5 protein, which inhibits (–)-strand DNA synthesis. Enough gene 2 protein is synthesized in the first 15 min to allow accumulation of RF DNA.

Materials

F^+ or Hfr *E. coli* strain
2× TY medium (*UNIT 1.1*)
Recombinant phage
20% glucose
1 mg/ml chloramphenicol in ethanol, freshly prepared
Sorvall SS-34 rotor or equivalent

1. Inoculate 20 ml of 2× TY medium plus 0.1 ml of 20% glucose with uninfected bacteria. Incubate in a 37°C shaking water bath until OD_{600} = 0.8 to 1.0.
2. Infect the cells with the recombinant phage at an MOI (see glossary in chapter introduction) of 20 to 50. Incubate 15 min at 37°C.
3. Add 0.3 ml of 1 mg/ml chloramphenicol in ethanol (final concentration, 15 µg/ml) to the culture. Incubate the culture an additional 2 hr.
4. Centrifuge 10 min at 4000 × *g* (6000 rpm) to harvest the cells. Prepare double-stranded DNA using the usual procedures for isolation of plasmid DNA (see *UNITS 1.6 & 1.7*).

PREPARING SINGLE-STRANDED DNA FROM PLASMIDS USING HELPER PHAGE

BASIC PROTOCOL

Cells containing plasmids with filamentous phage origins (usually the f1 origin) are infected with helper phage. The helper phage provides the gene 2 protein that drives the plasmid into the f1 mode of replication. The gene 2 protein nicks the (+)-strand origin of the plasmid and initiates rolling-circle replication, resulting in the production of single-stranded circles of the plasmid DNA. The helper phage also provides the DNA packaging and export functions. Single strands of the plasmid are packaged into phage coats and secreted into the supernatant. It is important to remember that only the (+) strand is packaged efficiently. Therefore, only the DNA strand of the insert that is in the same 5′→3′ orientation as the phage (+)-strand origin will be packaged.

Materials

F^+ or Hfr *E. coli* strain (Table 1.4.5) containing a plasmid (pUC118, pBS, or equivalent; see commentary)

2× TY medium (*UNIT 1.1*)

37°C shaking water bath and 65°C water bath

Sorvall SS-34 rotor or equivalent

1. Grow cells in 2× TY (containing an appropriate antibiotic) at 37°C to an OD_{600} of 0.1.

   A fresh overnight culture started from a single colony can be diluted 1:50 and grown to an OD_{600} of 0.1. Generally, 1- to 5-ml cultures will yield enough DNA for sequencing (typical yields are 0.2 to 1 μg plasmid single strands/ml culture).

2. Infect the cells at an MOI of 20. Some plasmids and bacterial strains seem to require addition of more phage (MOI of 50), while some give good yields with addition of less phage (MOI 5 to 10).

   When performing this protocol for the first time, it is helpful to try several different MOIs (1, 10, 20, and 50). Use the least amount of phage necessary to give a good yield of plasmid single strands because a portion of the input phage will always be recovered.

3. Grow the cells 4.5 hr at 37°C with vigorous shaking.

   For convenience, the infected cells can be grown overnight and the supernatant collected the following day. In many cases, this longer growth period will result in contamination of the supernatant with chromosomal DNA from lysed bacteria.

4. Centrifuge 10 min at 4000 × *g* (6000 rpm). Collect the supernatant and heat at 65°C for 15 min to kill any residual bacteria. Prepare single-stranded DNA from the supernatant (steps 4 to 13 of the second basic protocol, preparing single-stranded phage DNA from M13-derived vectors). Analyze the single-stranded DNA on an agarose gel (*UNIT 2.5*) with the helper phage serving as a control.

BASIC PROTOCOL

INTRODUCTION OF PHAGE DNA INTO CELLS

Both double-stranded and single-stranded vector DNA can be introduced into $CaCl_2$-treated competent bacteria by transfection, just as if the vector DNA molecules were plasmids. Single-stranded DNA usually yields about ten times fewer transformants than the same amount of double-stranded molecules.

Special applications may require vector DNA grown on an F- strain. The fact that phage DNA can be introduced into cells by transformation also makes it possible to produce phages from F- hosts. However, since phages produced by infection of these cells cannot infect neighboring cells, phages introduced into F- cells should always contain a drug-resistance marker so that transformed cells can be selected on antibiotic-containing plates.

Methods of transformation described in *UNIT 1.8* are used to introduce vector DNA into cells, with the following adaptations where appropriate.

1. If the vector contains a plasmid replicator and a drug-resistance gene, then simply select transformed cells on antibiotic-containing plates.
2. If the vector is able to form plaques, add 200 µl of noninfected cells at late log phase (OD_{600} = 0.6 to 0.8)—grown as in *UNIT 1.2*—to the plating-out mixture. Add 2.5 ml H top agar (*UNIT 1.1*), plate the mixture as a lawn on an H plate (*UNIT 1.1*), and incubate at 37°C until plaques appear (as in first basic protocol).

BASIC PROTOCOL

DETERMINING SIZE OF INSERTS IN SINGLE-STRANDED VECTORS

This method allows a quick comparison of a large number of viral DNAs without purification of the single-stranded DNA (Messing, 1983).

The size of inserts is estimated by comparing the mobility of DNA purified from insert-containing phage to the mobility of DNA from phages lacking inserts or containing inserts of known size. This procedure allows one to compare the sizes of single-stranded circular molecules that are several kilobases long; however, the resolution on agarose gels usually does not allow detection of inserts that are <300 bp long.

Materials

2% sodium dodecyl sulfate (SDS)
Loading buffer

1. Pipet 1.5 ml of an infected culture into a microcentrifuge tube.
2. Spin for 1 min. Remove 20 µl of the supernatant and mix with 1 µl of 2% SDS and 3 µl loading buffer. Repeat this process with supernatants of cultures of vector phage without inserts, and of other phage which contain inserts of known size.
3. Run these samples on a 0.7% agarose gel and analyze as described in *UNIT 2.5*. If desired, DNA can be transferred from gel to membrane and assayed with a radioactive probe, for example, to determine that the fragment has the right sequence as well as the right size.

DETERMINING INSERT ORIENTATION

SUPPORT PROTOCOL

Only one strand of the inserted DNA is contained in the phage's (+) strand and made into phage particles. This means that phage DNAs containing identical inserts in opposite orientations will hybridize with each other along the stretch of inserted DNA (see sketch 1.15A). The structures thus formed have different electrophoretic mobility than two unassociated single-stranded viral DNAs. This property of single-stranded phage vectors can be used to determine if two phages have an insert in opposite orientations (Howarth et al., 1981), which can be useful for sequencing both ends of a fragment of DNA.

Additional Materials

5 M NaCl

1. Mix 20 µl of two supernatants with 1 µl of 2% SDS in a microcentrifuge tube. In separate tubes mix 40 µl of each individual supernatant with 2 µl of 2% SDS.
2. Add 2 µl of 5 M NaCl to each of the three tubes and incubate 30 min at 60°C.
3. Run all three samples on a 0.7% agarose gel and visualize bands by staining with ethidium bromide as described in *UNIT 2.5*.

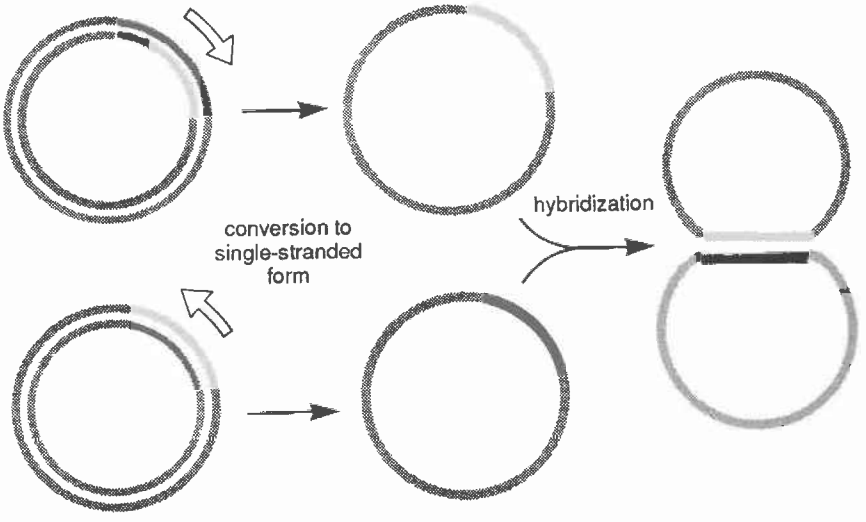
If many samples must be screened, it is possible to grow, lyse, and process recombinant infected cells in wells of microtiter plates.

It is also possible to determine the relative orientation of inserts in purified preparations of single-stranded DNA. To do this, mix 5 µl DNA from each of two independent plaques with 0.5 µl of 5 M NaCl. Incubate 30 min at 60°C, then analyze on an agarose gel as described above.

REAGENTS AND SOLUTIONS

Loading buffer

50% glycerol
0.2 M EDTA, pH 8.3
0.05% bromphenol blue



hybridization of two phages containing the same insert in opposite directions

Sketch 1.15A

COMMENTARY

Background Information

In M13 cloning vectors (Messing, 1983), a portion of the *E. coli lac* operon which bears a polylinker (a stretch of DNA which contains contiguous recognition sites for many different restriction enzymes) is inserted in the intergenic region (Messing et al., 1977). On an omega-fragment producing host, these vectors form blue plaques, and vectors with inserts form white plaques.

A plaque formed by M13 vectors is a zone of infected cells within a lawn of noninfected cells. Infected cells grow more slowly than uninfected cells. Plaques made by phage that contain inserts are usually smaller than those made by wild-type phage. This is because phage DNA which contains inserts is replicated more slowly, and it takes longer for cells infected with those phages to produce progeny plaques. Many people prefer to store the recombinant phages not as plaques but as purified single-stranded DNA.

Other single-stranded cloning vectors contain the origins of replication of phages like f1. These vectors usually also contain the pBR322 plasmid origin of replication, a drug resistance coding gene, and a polylinker inserted in frame into the portion of the *lacZ* gene coding for the alpha peptide. Most of these vectors lack gene 2, or the other phage genes necessary to form single-strand phage particles.

In cells these vectors are double-stranded and replicate using the pBR322 origin of replication. However, when cells are infected with wild-type helper phages, the plasmids replicate using the phage origin of replication, and single-stranded copies of the plasmid are encapsidated into phage filaments, which are then secreted into the culture medium along with copies of the helper. Contamination with helper phage does not interfere with most applications because the helper does not contain genes encoding *lacZ* or ampicillin resistance, nor is it likely to contain any piece of nucleic acid used as a hybridization probe.

Perhaps the most common application of these vectors is in sequencing using the dideoxy method (*UNIT 7.4*). In this procedure, small fragments of DNA from a plasmid or lambda-derived vector are subcloned into M13mp vectors. Vectors containing the inserts are identified by the fact that they form colorless plaques on a lawn of omega-fragment producing cells growing on Xgal + IPTG plates (see *UNIT 1.4*).

For further background information see lifecycle of filamentous phages, *UNIT 1.14*, and key references listed there.

Critical Parameters

Isolating single M13-derived vectors. Plates should be used immediately after plaques have appeared. Phages should be freshly plated every time a single plaque is to be picked for DNA amplification. Phages diffuse, and use of an old plate can result in cross contamination. Cells to be infected and lawns of infected cells must be grown at 37°C. If cells are grown at temperatures below 34°C, sex pili do not form and phages cannot infect.

Preparing single-stranded phage DNA from M13-derived vectors. It is critical to remove PEG from the preparations, since traces of PEG inhibit the activity of many DNA polymerases. Contaminated templates can be extracted with chloroform prior to ethanol precipitation. However if step 7 (of the first basic protocol) has been followed carefully, chloroform extraction is not necessary.

Preparing replicative-form (RF) DNA. Typical yields of double-stranded DNA from chloramphenicol-treated cells are 50 to 200 µg per 20-ml culture depending on how well the particular recombinant phage grows. Too much chloramphenicol will result in reduced yields, while too little will result in contamination with single-stranded DNA.

Sometimes after the cesium chloride gradient is equilibrated, a third band appears between the plasmid DNA band (lowest) and the genomic DNA band (highest). This is made of single-stranded DNA and should be left behind.

Preparing single-stranded DNA from plasmids using helper phage. Start with a single colony of male *E. coli* strain that is harboring a recombinant plasmid containing a filamentous phage replication origin such as pUC118 (Viera and Messing, 1987) or pBS (Stratagene).

The strain of helper phage used must be appropriate for the experiment. Several helper phages such as IR-1 (Enea and Zinder, 1982), R408 (Russell et al., 1986), M13K07 (Veira and Messing, 1987), and VCSM13 (derived from M13K07; Stratagene) are available. The phages IR-1 and R408 are more stable than VCSM13 and M13K07, but the latter phages contain a kanamycin-resistance gene that aids in selection. The helper phages VCSM13 and R408 were designed to favor the production of plasmid single strands. VCSM13 has a phage (+)-strand replication origin with

a defective replication "enhancer" sequence. Therefore, it doesn't compete as well for the gene 2 protein and hence favors the production of plasmid single strands. In contrast, the helper phage R408 has a defective packaging signal (see *UNIT 1.14*) causing the plasmid single strands to be preferentially packaged into phage particles. Regardless of the helper phage used, the single-stranded DNA prepared from the supernatant will contain some DNA from the helper phage. This is usually not a problem for DNA sequencing because a primer can be chosen that is specific for the plasmid single strands.

Introduction of phage DNA into cells. Transformation of $CaCl_2$-treated competent bacteria is carried out as described in *UNIT 1.8*. If the vector forms plaques, it is not necessary to phenotypically express, and the cells can be plated out immediately following the heat shock step. Using F^- competent cells is a common mistake, since the recombinant phage will not form plaques on a lawn of F^- cells. However, F^- competent cells can be used if they are diluted with uninfected F^+ cells following transformation and plated together. In this case, the F^- cells will produce phage that will make plaques on the mixed lawn. As long as the F^+ cells are in abundance, plaques will be seen.

Determining size of inserts in single-stranded vectors. Single-stranded DNA is analyzed from a phage supernatant after extraction from the phage particle. It is important to vortex the phage with the SDS to remove the coat proteins from the phage DNA. Failure to do so will result in incomplete extraction and smearing of the DNA bands. If the culture supernatant contains bacteria or chromosomal DNA from lysed bacteria, the phage DNA will be contaminated and other DNA bands will be visible. If this is a recurrent problem, the position of these extra bands can be determined by running an uninfected culture supernatant as a control.

Time Considerations

Plaques can be seen after ~4 hr. The color of plaques can be determined after a few more hours of incubation.

When preparing single-stranded phage DNA, incubation and processing samples are performed the same day. The only limiting factor is the number of samples to process at the same time. If there are too many, it is possible to store the supernatants (step 5, first basic protocol) at 4°C for 24 hr and then recentrifuge before proceeding to the next step.

Preparing single-stranded DNA with helper phage requires a shorter incubation period (4.5 hr) after inoculation with the phage. Preparing RF DNA from chloramphenicol-treated cells can be accomplished within one day after the incubation. An additional day may be required if the sample is to be purified on a CsCl gradient.

Introducing phage DNA into cells, determining insert size, and determining insert orientation each takes ~2 hr.

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Reviews biology of non-M13–derived single-stranded vectors.

Howarth et al. 1981. See above.

Describes techniques for determining insert orientation.

Messing, J. 1983. See above.

Maintenance, propagation, and titration of filamentous phage vectors are extensively described.

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PROTEIN EXPRESSION

16

continued

continued

continued

CHAPTER 16
Protein Expression

INTRODUCTION

Protein expression, as used in this chapter, refers to the directed synthesis of large amounts of desired proteins. In early applications, molecular biologists interested in obtaining large amounts of prokaryotic regulatory proteins arranged their synthesis in large amounts, a process that came to be called overproduction, expression, or overexpression. These early techniques used genetic manipulations to select in vivo recombination events that inserted the desired gene into bacteriophages. Later, as it was developed, recombinant DNA technology was used to create phages and plasmids in vitro, which directed the synthesis of large amounts of the products of cloned genes.

This chapter describes methods to express proteins. In all these methods, a gene whose product is to be expressed is introduced into a plasmid or other vector, and that vector is introduced into living cells. Typical expression vectors contain promoters that direct the synthesis of large amounts of mRNA corresponding to the gene. They may also include, for example, sequences that allow their autonomous replication within the host organism, sequences that encode genetic traits that allow cells containing the vectors to be selected, and sequences that increase the efficiency with which the mRNA is translated.

Section I (*UNITS 16.1-16.8*) describes techniques for expressing proteins in *E. coli*. *UNIT 16.1* contains an introduction to *E. coli* expression. *UNIT 16.2* describes the use of T7 vectors, in which synthesis of large amounts of foreign gene products is directed by the phage T7 gene 10 promoter, which uses T7 RNA polymerase. This polymerase transcribes the gene 10 promoter so efficiently that it uses up most of the ribonucleotide triphosphates in the cell and drastically inhibits transcription of genes by the host polymerase. *UNIT 16.3* describes the use of p_L-derived vectors and their appropriate host strains. These vectors carry the powerful bacteriophage p_L promoter and take advantage of a number of other useful aspects of phage lambda biology. The next units contain techniques for expression of fusion proteins in which the expressed protein carries an additional stretch of amino acids at its N terminus to aid its expression and purification. *UNIT 16.4* introduces the concept of fusions and provides methods for cleavage of fusion proteins. *UNITS 16.5, 16.6, 16.7 & 16.8* describe techniques for expressing *lacZ* protein (β-galactosidase), *trpE* protein, maltose-binding protein, glutathione-*S*-transferase, and thioredoxin fusions.

Section II (*UNITS 16.9-16.11*) describes the use of the baculovirus system. In this system, genes for proteins to be expressed are inserted into an insect virus in lieu of a highly expressed dispensable gene. The foreign protein is produced by growing the recombinant virus in cultured insect cells. *UNIT 16.9* introduces the system. *UNIT 16.10* describes how to grow the cultured insect cells and viral stocks and how to isolate recombinant baculoviruses and use them to produce the desired protein. Finally, *UNIT 16.11* describes the optimization of protein expression, first on a small scale, then maximized for large-scale production, and then provides instructions for purifying the recombinant proteins using GST- and His-tag systems.

Sections III and IV (*UNITS 16.12-16.20*) describe techniques for expressing proteins in mammalian cells. *UNIT 16.12* introduces the general issues. *UNIT 16.13* describes expression using COS cell vectors. In this approach, vectors containing the gene to be expressed are

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transiently transfected into COS cells, which constitutively produce SV40 large T antigen. COS cell vectors contain an SV40 replication origin; when they are transfected into COS cells, they replicate, and protein is expressed from mRNA synthesized by hundreds of copies of the vectors. *UNIT 16.14* describes expression of proteins by specially constructed CHO cell lines. These lines contain integrated constructions to direct the synthesis of the mammalian protein; the constructions carry either the dihydrofolate reductase or the glutamine synthetase gene, whose products confer drug resistance. Lines that carry increased numbers of the constructs are obtained by selecting cell lines that grow in increasing drug concentrations of methotrexate. Once selected, these lines are permanent reagents, which can be stored frozen and used to produce the protein whenever desired.

Expression of proteins using viral vectors is presented in *UNITS 16.15-16.20*. *UNITS 16.15-16.19* outline how to construct recombinant vaccinia viruses and characterize their products. *UNIT 16.20* describes how to carry out expression using Semliki Forest Virus (SFV) vectors.

The latest section in this chapter will discuss specialized expression systems. The first in Section V, *UNIT 16.21*, describes the pTET autoregulatory system. Tetracycline-regulated expression systems were developed to overcome some of the obstacles seen in other inducible systems, such as toxicity of inducing agents or high uninduced background levels of expression. This unit describes protocols for using a modified tetracycline-regulated system in which a transcriptional activator drives expression of itself and a target gene in cultured cells.

All expression techniques have advantages and disadvantages that should be considered in choosing which one to use. *E. coli* expression techniques are probably the most popular: the organism is already used by most investigators, the techniques necessary to express usable amounts of protein are relatively simple, the amount of time necessary to generate an overexpressing strain is very short, and a familiarity with standard recombinant DNA techniques is all that is necessary to begin pilot expression experiments. *E. coli* has other advantages that have made it widely used for expression of commercially important proteins: it is cheap to grow, and the vast body of knowledge about it has made it possible to tinker intelligently with its genetics and physiology, so that strains producing 30% of their total protein as the expressed gene product can often be obtained. However, expression in *E. coli* does have some disadvantages. First, eukaryotic proteins expressed in *E. coli* are not properly modified. Second, proteins expressed in large amounts in *E. coli* often precipitate into insoluble aggregates called "inclusion bodies," from which they can only be recovered in an active form by solubilization in denaturing agents followed by careful renaturation. Third, it is relatively difficult to arrange the secretion of large amounts of expressed proteins from *E. coli*, although it has often been possible to secrete small amounts into the periplasmic space and to recover them by osmotic shock.

The baculoviral expression system has a number of advantages that have contributed to its recent popularity: proteins are almost always expressed at high levels; expressed proteins are usually expressed in the proper cellular compartment (that is, membrane proteins are usually localized to the membrane, nuclear proteins to the nucleus, and secreted proteins secreted into the medium); and the expressed protein is often properly modified. Expression using baculoviral vectors also has some drawbacks: the techniques to grow and work with the virus are still not very widely used and may be difficult for the beginner; the expressed proteins are not always properly modified; and, even for the sophisticated, generation of a recombinant baculovirus to express a given protein still takes a considerable amount of work.

Compared with the above systems, all mammalian expression techniques have certain advantages, particularly for the expression of higher eukaryotic proteins: expressed

proteins are usually properly modified, and they almost always accumulate in the correct cellular compartment. Generally speaking, mammalian expression techniques are more difficult, time-consuming, and expensive than those used to express proteins in *E. coli*, and they are much more difficult to perform on a large scale; but they are quite practical for small- and medium-scale work by investigators already familiar with mammalian cell culture techniques. The three procedures described here are appropriate for slightly different applications. The COS cell and virus procedures are suitable for rapid small- and medium-scale protein production. The CHO cell procedure, by contrast, is more appropriate for large-scale protein production. Although it can take months to generate a highly amplified CHO cell line, such a line can be stored frozen in liquid nitrogen, and used indefinitely to express the protein, consistently and without the need for continual production of viral stocks.

Roger Brent

EXPRESSION OF PROTEINS IN *ESCHERICHIA COLI*

Overview of Protein Expression in *E. coli*

The study of *Escherichia coli* during the 1960s and 1970s made it the best understood organism in nature (Chapter 1). Today's recombinant DNA technology is a direct extension of the genetic and biochemical analyses carried out at that time. Even before the advent of molecular cloning, genetically altered *E. coli* strains were used to produce quantities of proteins of scientific interest. When cloning techniques became available, most cloning vectors utilized *E. coli* as their host organism. Thus, it is not surprising that the first attempts to express large quantities of proteins encoded by cloned genes were carried out in *E. coli.*

E. coli has two characteristics that make it ideally suited as an expression system for many kinds of proteins: it is easy to manipulate and it grows quickly in inexpensive media. These characteristics, coupled with more than 10 years' experience with expression of foreign genes, have established *E. coli* as the leading host organism for most scientific applications of protein expression.

Despite a growing literature describing successful protein expression from cloned genes, each new gene still presents its own unique expression problems. No one, and certainly no laboratory manual, can provide a set of methods that will guarantee successful production of every protein in a useful form. Nevertheless, the vast body of accumulated knowledge has led to a general approach that often helps to solve specific expression problems. This unit introduces general considerations and strategies, while subsequent units (*16.2-16.7*) describe procedures that can be applied to specific expression problems.

GENERAL STRATEGY FOR GENE EXPRESSION IN *E. COLI*

The basic approach used to express all foreign genes in *E. coli* begins with insertion of the gene into an expression vector, usually a plasmid. This vector generally contains several elements: (1) sequences encoding a selectable marker that assure maintenance of the vector in the cell; (2) a controllable transcriptional promoter (e.g., *lac, trp,* or tac) which, upon induction, can produce large amounts of mRNA from the cloned gene; (3) translational control sequences, such as an appropriately positioned ribosome-binding site and initiator ATG; and (4) a polylinker to simplify the insertion of the gene in the correct orientation within the vector. Once constructed, the expression vector containing the gene to be expressed is introduced into an appropriate *E. coli* strain by transformation (*UNIT 1.8*).

SPECIFIC EXPRESSION SCENARIOS

Although this general approach—insertion of the gene of interest into an expression vector followed by transformation in *E. coli*—is common to all expression systems, specific procedures differ greatly. When choosing a procedure, it is helpful to consider the final application of the expressed protein, as this often dictates which expression strategy to use (*UNIT 16.4A*).

Antigen Production

If the goal is to use the expressed protein as an antigen to make antibodies, several approaches are available to make protein reliably and to allow for rapid purification of the antigen. The two best approaches are synthesis of fusion proteins with specific "tag" sequences that can be retrieved by affinity chromatography (*UNITS 16.5, 16.6, 16.7 & 16.8;* see also *UNIT 10.11B*) and synthesis of the native protein, or a fragment of it, under conditions that cause it to precipitate into insoluble inclusion bodies (*UNITS 16.4A & 16.6*). These inclusion bodies can be purified sufficiently by differential centrifugation so preparative denaturing polyacrylamide gel electrophoresis (*UNIT 10.2*) will yield an isolated band that can be cut out and crushed, or electroeluted (*UNIT 10.5*), to provide antigenic material for injection into an animal.

Biochemical or Cell Biology Studies

If the goal is to use the expressed protein as a reagent in a series of biochemical or cell biology experiments, other considerations are relevant. In this case, the authenticity of the protein's function (e.g., high-specific-activity enzyme, binding protein, or growth factor) is

very important, while the ease of preparing the protein matters less. For this application, it is possible to express the protein as a fusion protein containing a specific protease-sensitive cleavage site so the N-terminal peptide tail can be removed easily, leaving only the native amino acid sequence (*UNITS 16.4, 16.6, 16.7 & 16.8*). Alternatively, direct expression vectors of the type described in *UNITS 16.2 & 16.3* may be used to produce the authentic primary sequence. When expressed, the protein may be soluble and active, as is the case with many intracellular enzymes. If it is insoluble, as is the case for many secreted growth factors when they are made cytoplasmically in *E. coli*, it may be necessary to isolate inclusion bodies, solubilize the protein using denaturing agents, and refold the protein. Refolding is usually not too difficult when the protein is of moderate size (Marston and Hartley, 1990). Whether the protein is expressed in a soluble form or whether it requires refolding, its integrity can usually be checked by specific enzyme assays or by bioassays.

Structural Studies

If the goal is to do structural studies of the expressed protein, the greatest constraints are imposed on the expression system. Because it is nearly impossible to show that a protein of unknown structure has been precisely refolded after denaturing, the protein must generally be made in a soluble form so its purification does not require a denaturation/renaturation step. Usually, the soluble form of the protein—either intracellular or secreted—must be made in strains and by induction protocols that minimize proteolytic degradation.

Soluble expression of most eukaryotic proteins is best achieved with systems that allow induction of synthesis without changing the temperature; for example, by inducing transcription from the *trp* (Edman et al., 1981; de Boer et al., 1983) or tac (de Boer et al., 1983) promoters. Maximum accumulation of soluble product is best achieved by testing expression in several strains and at several temperatures, and picking the combination that works best. This is an active area of research at present (Schein, 1989); the rules are not yet understood, so little more than trial and error can be recommended.

TROUBLESHOOTING GENE EXPRESSION

Once an expression strategy has been chosen and the gene is introduced into an appropriate expression vector, several strains of *E. coli* should be transformed with the vector and protein production should be monitored. Ideally, the protein of interest will be produced in an active form and in sufficient amounts to allow its isolation. Often, however, the protein will be made either in very small amounts or in an insoluble form, or both. If this happens, there are various approaches that may correct the problem.

If not enough protein is produced:

1. Reconstruct the 5′-end of the gene, maximizing its A+T content while preserving the protein sequence it encodes. This may reduce secondary structure within the mRNA (DeLamarter et al., 1985), or it may alter an as yet undefined parameter of the reaction. Regardless of the underlying cause, this procedure usually increases translation efficiency.

2. Determine if a transcriptional terminator is present. If the vector does not have a transcriptional terminator downstream from the site at which the gene is inserted, put one in. This often aids expression, probably by increasing mRNA stability and by decreasing nucleotide drain on the cell.

3. Examine the sequence of the cloned gene for codons used infrequently in *E. coli* genes. These so-called rare codons are usually not a rate-limiting problem, but if four or more happen to occur contiguously, they can reduce expression significantly (Robinson et al., 1984), perhaps by causing ribosomes to pause. Ribosomal pausing can uncouple transcription from translation, leading to premature termination of the message. Even if transcription proceeds normally, the mRNA 3′ to the stalled ribosomes can be exposed to degradation by host ribonucleases, reducing its stability. Thus, if stretches of rare codons are found, they should be altered to codons more favorable to high expression in *E. coli*.

If enough protein is produced, but it is insoluble when the application requires it to be active and soluble:

1. Vary the growth temperature. As mentioned above, many proteins are more soluble at lower than at higher temperatures (Schein and Noteborn, 1988). On the other hand, some enzymes have a higher specific activity when made at temperatures >37°C (J. McCoy and P. Schendel, unpub. observ.). *E. coli* can synthesize proteins at temperatures ranging from 10° to 43°C, so trying expression at different temperatures is often worthwhile.

2. Change fermentation conditions. Many proteins contain metals as structural and catalytic cofactors. If the protein is being made faster than metals can be transported into the cell, the apoprotein without its metal cofactor will accumulate. This apoprotein will not fold correctly and will likely be insoluble. At the very least, the average specific activity of the expressed protein will be lower than expected. Different media and metal supplements can be tested and the best combination used. Clearly, if there is information about the metal content of the protein, these supplements can be designed more rationally. If no information is available, a more random approach must be tried.

3. Alter the rate of expression by using low-copy-number plasmids. This can be done by using the pACYC family (Chang and Cohen, 1978) or using single-copy chromosomal inserts of the cloned gene into a suitable target gene (Hamilton et al., 1989). Such reductions in gene dosage often reduce the final yield of protein, but the slower kinetics of synthesis they afford can sometimes result in production of soluble proteins.

To restate the obvious, protein expression is an inexact science at present. However, most proteins can be made in *E. coli* in a form that is useful for a variety of functions. The procedures employed are relatively quick and uncomplicated, and the rewards for success are great.

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Expression Using the T7 RNA Polymerase/Promoter System

This unit describes the expression of genes by placing them under the control of the bacteriophage T7 RNA polymerase. This approach has a number of advantages compared to approaches that rely on *E. coli* RNA polymerase. First, T7 RNA polymerase is a very active enzyme: it synthesizes RNA at a rate several times that of *E. coli* RNA polymerase and it terminates transcription less frequently; in fact, its transcription can circumnavigate a plasmid, resulting in RNA several times the plasmid length in size. Second, T7 RNA polymerase is highly selective for initiation at its own promoter sequences and it does not initiate transcription from any sequences on *E. coli* DNA. Finally, T7 RNA polymerase is resistant to antibiotics such as rifampicin that inhibit *E. coli* RNA polymerase, and consequently, the addition of rifampicin to cells that are producing T7 RNA polymerase results in the exclusive expression of genes under the control of a T7 RNA polymerase promoter (hereafter referred to as p_{T7}).

To use the two-plasmid p_{T7} system, it is necessary to clone the gene to be expressed into a plasmid containing a promoter recognized by the T7 RNA polymerase. The gene is then expressed by induction of T7 RNA polymerase. The gene for T7 RNA polymerase is present on a second DNA construction. This second construction can either permanently reside within the *E. coli* cell (basic protocol), or can be introduced into the cell at the time of induction by infection with a specialized phage, such as an M13 vector (mGP1-2; Tabor and Richardson, 1987) or a λ vector (CE6; Studier et al., 1990) containing the T7 RNA polymerase gene (second alternate protocol).

In the basic protocol, two plasmids are maintained within the same *E. coli* cell. One (the expression vector) contains p_{T7} upstream of the gene to be expressed. The second contains the T7 RNA polymerase gene under the control of a heat-inducible *E. coli* promoter. Upon heat induction, the T7 RNA polymerase is produced and initiates transcription on the expression vector, resulting in turn in the expression of the gene(s) under the control of p_{T7}. If desired, the gene products can be uniquely labeled by carrying out the procedure in minimal medium, adding rifampicin to inhibit the *E. coli* RNA polymerase, and then labeling the proteins with [^{35}S]methionine (first alternate protocol).

EXPRESSION USING THE TWO-PLASMID SYSTEM

BASIC PROTOCOL

The gene to be induced is subcloned into an expression vector containing p_{T7}. Two series of vectors have been developed for this purpose—the pT7 series (Fig. 16.2.1) and the pET series (Studier et al., 1990); see commentary for discussion of choice of vector. The plasmid containing the introduced gene is then used to transform an *E. coli* strain already containing the plasmid pGP1-2 (Fig. 16.2.2). pGP1-2 contains the gene for T7 RNA polymerase under the control of the λ p_L promoter that is repressed by a temperature-sensitive repressor (*c*I857). pGP1-2 contains a p15A origin of replication that is compatible with the ColE1 origin of replication on the expression vector. The two plasmids are maintained in the same cell by selection with kanamycin (pGP1-2) and ampicillin (the expression vector).

Cells containing the two plasmids are grown for several hours at 30°C and then the gene for T7 RNA polymerase is induced by raising the temperature to 42°C. The production of T7 RNA polymerase in turn induces expression of the genes under the control of p_{T7}. (Rifampicin can be subsequently added to inhibit transcription by *E. coli* RNA polymerase, although this is usually not necessary since T7 RNA polymerase becomes responsible for most of the transcription even in the absence of rifampicin.) After expression

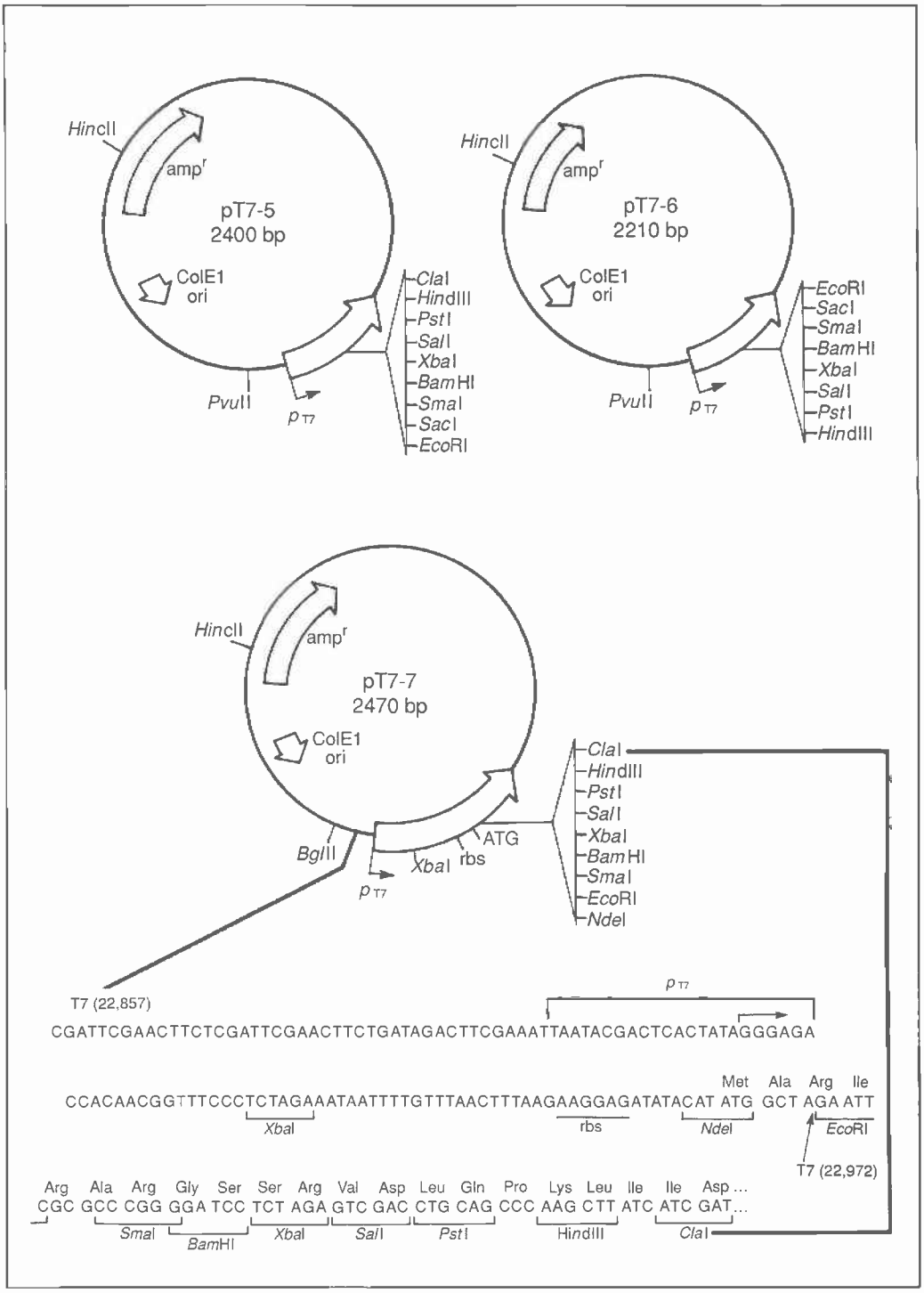

Figure 16.2.1 pT7-5, pT7-6, and pT7-7. pT7-5, pT7-6, and pT7-7 are cloning vectors that contain a T7 promoter and are used to express genes using T7 RNA polymerase. All three vectors contain a T7 RNA polymerase promoter, the gene encoding resistance to the antibiotic ampicillin and the ColE1 origin of replication. pT7-7 has a strong ribosome-binding site (rbs) and start codon (ATG) upstream of the polylinker sequence; the sequence of this region is shown below the map of pT7-7. pT7-5 and pT7-6 lack any ribosome-binding site upstream of the polylinker sequence and consequently are only useful when expressing genes that already contain the proper control sequences. pT7-5, pT7-6, and pT7-7 were constructed by S. Tabor and are derivatives of pT7-1 described in Tabor and Richardson (1985).

of the genes at 37°C, the cells are harvested and the induced proteins are analyzed. An alternative approach is to induce T7 RNA polymerase with IPTG rather than by heat induction. In this method, the expression plasmid containing p_{T7} can be placed into *E. coli* BL21 (DE3), which contains the gene for T7 RNA polymerase on the *E. coli* chromosome under the control of the *lac* promoter (Studier and Moffatt, 1986; Studier et al., 1990).

Materials

pT7-5, pT7-6, or pT7-7 vectors (available from author)
E. coli JM105, DH1, or equivalent (Table 1.4.5)
LB plates and medium containing 60 µg/ml ampicillin (*UNIT 1.1*)
E. coli K38 or equivalent (Table 1.4.5)
pGP1-2 (available from author)
LB plates and medium containing 60 µg/ml kanamycin (*UNIT 1.1*)
LB plates and medium containing 60 µg/ml ampicillin plus 60 µg/ml kanamycin (*UNIT 1.1*)
Cracking buffer
Sorvall SS-34 or GS-3 rotor or equivalent

Additional reagents and equipment for subcloning DNA fragments (*UNITS 1.4 & 3.16*), transformation of competent *E. coli* cells (*UNIT 1.8*), minipreps of plasmid DNA (*UNIT 1.6*), restriction mapping (*UNITS 3.1-3.3*), and SDS-PAGE (*UNIT 10.2*).

1. Subclone the fragment containing the gene to be expressed into pT7-5, pT7-6, or pT7-7. Transform a standard *E. coli* strain (e.g., JM105 or DH1); this strain should *not* carry a plasmid that directs synthesis of T7 RNA polymerase (i.e., pGP1-2). Plate the transformants on LB/ampicillin plates and grow overnight at 37°C.

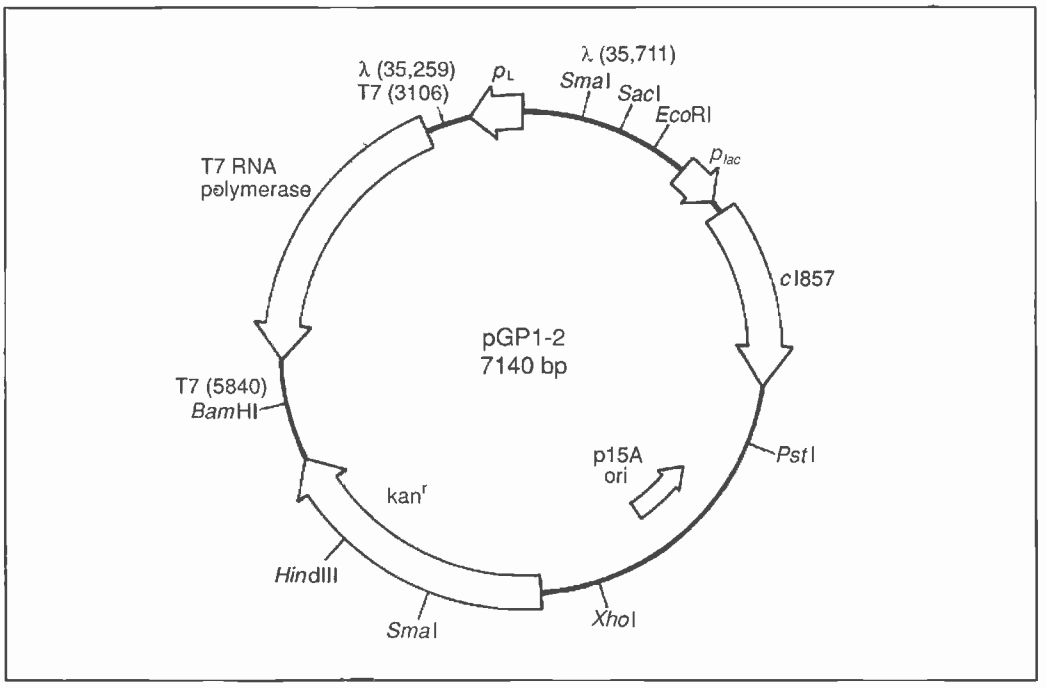

Figure 16.2.2 pGP1-2. pGP1-2 enables T7 RNA polymerase to be produced by heat induction in any *E. coli* host. pGP1-2 contains the gene for T7 RNA polymerase under the control of the λ p_L promoter. It also contains the gene for the λ repressor (cI857) that is expressed under the control of *E. coli* p_{lac} promoter. This repressor inhibits transcription from the λ p_L promoter at low temperature (30°C); however, at high temperature (42°C) it is inactivated, resulting in induction of the p_L promoter, that in turn results in induction of the T7 RNA polymerase. pGP1-2 also contains the gene encoding resistance to the antibiotic kanamycin, and the p15A origin of replication. pGP1-2 is described in Tabor and Richardson (1985).

It is important to first transform the plasmid into a strain that contains no T7 RNA polymerase, in case small amounts of the gene product are toxic to the cell (see critical parameters for discussion on toxic genes).

2. Grow individual transformants in LB/ampicillin medium at 37°C and obtain plasmid DNA by a miniprep procedure. Confirm that the gene has been correctly inserted by restriction mapping.

3. Transform *E. coli* K38 with pGP1-2, plate on LB/kanamycin plates, and grow overnight at 30°C. Grow an individual *E. coli* K38/pGP1-2 transformant in LB/kanamycin medium at 30°C.

   Colonies take ~24 hr to appear on plates at 30°C. E. coli K38/pGP1-2 can be stored in the absence of the plasmid containing p_{T7} as a glycerol stock at −80°C (see commentary).

4. Transform the vector containing the gene to be expressed under the control of p_{T7} into *E. coli* K38/pGP1-2 grown in LB/kanamycin medium. Plate the transformants (containing both plasmids) on LB/ampicillin/kanamycin plates and grow overnight at 30°C.

   Cells may be heat-shocked during transformation; the T7 RNA polymerase gene, under the control of a heat-inducible promoter, is not induced by this brief heating step.

   As a control, transform E. coli K38/pGP1-2 with the parent p_{T7} vector (without an insert). If the transformation efficiency of the vector containing the insert is significantly lower (by more than a factor of 50) than that of the parent vector, the gene product may be toxic to E. coli cells. This toxicity arises from background expression of the gene product by basal levels of T7 RNA polymerase. In this situation, the transformants that do arise invariably contain deletions or other mutations in one of the two plasmids, and the desired gene product will not be produced. If the expression of the inserted gene is toxic, it is necessary to use an alternative strategy for the repression and induction of the T7 RNA polymerase gene (see discussion on toxic genes in critical parameters).

5. Pick a single *E. coli* colony that contains the two plasmids with a sterile toothpick or pipet. Inoculate it into 5 ml LB/ampicillin/kanamycin medium and grow overnight at 30°C.

6. Dilute the overnight culture of cells 1:40 into fresh LB/ampicillin/kanamycin medium and grow several hours at 30°C to an $OD_{590} \cong 0.4$.

   The size of the culture will depend on the amount of cells needed. For an analytical preparation, use ~1 ml of cells.

7. Induce the gene for T7 RNA polymerase by raising the temperature to 42°C for 30 min, which in turn induces the genes under the control of p_{T7}.

   To obtain consistent results, raise the temperature relatively quickly. If small cultures (~1 ml) are being induced, place the cultures into a 42°C water bath. For larger cultures (~500 ml), place the flask under hot tap water until the temperature of the media reaches 42°C (measured by inserting a thermometer wiped with ethanol into the flask). Once the cells reach 42°C, continue incubating at 42°C for 30 min.

   The E. coli RNA polymerase can be inhibited by adding rifampicin to a final concentration of 200 µg/ml; when used, it should be added after T7 RNA polymerase has been induced at 42°C for 30 min. Although rifampicin reduces the background of host proteins being expressed, in general it does not significantly increase the final accumulation of gene products, and in some cases it decreases the final yield. Thus, as a general rule, rifampicin is only added to cells when the plasmid-encoded proteins are being uniquely labeled with [^{35}S]methionine (see first alternate protocol).

8. Reduce temperature to 37°C and grow the cells an additional 90 min with shaking.

9. Harvest the cells by centrifuging and discarding the supernatant. For 1-ml cultures, microcentrifuge 20 sec at 10,000 rpm (14,000 × *g*), room temperature. For 2-ml to 100-ml cultures, centrifuge 5 min in a Sorvall SS-34 rotor at 5000 rpm (3000 × *g*), 4°C. For >100-ml cultures, centrifuge 10 min in a Sorvall GS-3 rotor at 5000 rpm (4000 × *g*), 4°C.

10. To analyze the induced proteins by SDS-PAGE, resuspend the equivalent of 1.0 ml of cells in 0.1 ml cracking buffer. Heat at 100°C for 5 min immediately prior to loading a 20-µl aliquot of each sample onto an SDS-polyacrylamide gel (*UNIT 10.2*). To analyze the cells for an induced enzymatic activity, prepare an appropriate cell extract from ~10 ml of cells.

    One example of the preparation of an extract for the purification of T7 RNA polymerase is described in Tabor and Richardson (1985).

SELECTIVE LABELING OF PLASMID-ENCODED PROTEINS

ALTERNATE PROTOCOL

Plasmid-encoded proteins under the control of a p_{T7} (see basic protocol) can be exclusively labeled by inducing the T7 RNA polymerase in cells growing in minimal medium, inhibiting the host *E. coli* RNA polymerase with rifampicin, and labeling the newly synthesized proteins with [^{35}S]methionine. This procedure provides an attractive alternative to maxicells or minicells for labeling of plasmid-encoded proteins (Dougan and Sherratt, 1977; Sancar et al., 1981).

Additional Materials

M9 medium (*UNIT 1.1*) without and with 5% (vol/vol) of 18 amino acid mixture
20 mg/ml rifampicin in methanol (e.g., Sigma #R-3501; store in dark at 4°C for 2 weeks; Table 1.4.1)
10 mCi/ml [^{35}S]methionine (>800 Ci/mmol) diluted 1:10 in M9 medium
Fluorographic enhancing agent (e.g., Enlightning from Du Pont NEN or Amplify from Amersham)

1. Repeat steps 3 to 6 of the basic protocol (using the T7-promoter expression plasmid obtained from steps 1 and 2 of the basic protocol).

    An alternative to the use of LB/ampicillin/kanamicin medium for growing cells is M9 medium containing 25 µg/ml ampicillin and 25 µg/ml kanamycin, and any required nutrients. The addition of one part in twenty of the 18 amino acid mixture (0.1% stock, 0.005% final concentration) stimulates the growth of cells in M9 medium without interfering with the subsequent labeling of the proteins with [^{35}S]methionine. Note that to grow in this medium, the E. coli strain must be Cys$^+$ and Met$^+$.

2. When $OD_{590} \cong 0.4$, remove 1 ml of cells, microcentrifuge 10 sec, and discard supernatant.

3. Wash cell pellet with 1 ml M9 medium, microcentrifuge 10 sec at room temperature, and discard supernatant.

    Washing the cells after growth in LB medium is very important in order to remove the unlabeled methionine present in LB medium that otherwise dilutes the [^{35}S]methionine during labeling.

4. Resuspend cell pellet in 1 ml M9 medium containing 18 amino acid mixture. Grow cells 60 min at 30°C with shaking.

    A time of 30 to 180 min is adequate for adapting cells to M9 medium. Although the OD_{590} may not increase significantly during this step, induction of T7 RNA polym-

erase and efficient labeling of the plasmid-encoded proteins will occur even in the absence of apparent cell growth.

5. Induce the gene for T7 RNA polymerase by placing the cells in a 42°C water bath for 20 min.

6. Add 20 mg/ml rifampicin to 200 μg/ml final. Keep cells at 42°C for an additional 10 min after adding rifampicin.

   It is important to incubate the cells at 42°C for an additional 10 min after adding rifampicin, since rifampicin is more effective at inhibiting expression of host proteins at 42°C, possibly because the cells are more permeable to it at this temperature. The temperature of the cells is subsequently reduced for the labeling since in general the labeling is less efficient at 42°C than at 30° or 37°C.

7. Shift cells to a 30°C water bath for an additional 20 min. Remove 0.5 ml of cells for labeling with [^{35}S]methionine.

   The other 0.5 ml can be used to label the cells at a later time point (e.g., after an additional 30 min) in order to follow the duration of protein synthesis.

8. Label newly synthesized proteins by adding 10 μl (10 μCi) diluted [^{35}S]methionine to 0.5 ml of cells and incubating for 5 min at 30°C.

9. Microcentrifuge cells 10 sec and discard supernatant. (*CAUTION*: the supernatant is radioactive; discard properly.) Resuspend cell pellet in 100 μl cracking buffer.

10. Heat samples to 100°C for 5 min. Load a 20-μl aliquot onto an SDS-polyacrylamide gel and electrophorese (*UNIT 10.2*).

11. Treat the gel with a fluorographic-enhancing agent by soaking it in the fluor for 30 min. Dry the gel under vacuum 2 hr at 65°C and autoradiograph (*APPENDIX 3*).

    A 1-hr exposure should be adequate to visualize most proteins induced with this system.

    To determine whether the plasmid-encoded proteins are susceptible to proteases in the E. coli cell, prepare and induce the cells as described above; however, reduce the duration of the labeling step to 1 min (step 8), and follow this with a chase of nonradioactive methionine at 0.5% final concentration. Remove an aliquot for analysis both immediately prior to the chase, and after a chase reaction of 5, 15, and 60 min. After removing each aliquot, immediately pellet the cells by centrifugation, resuspend in cracking buffer, and heat the aliquot to 100°C for 5 min to inactivate the proteases. Analyze as in step 10.

ALTERNATE PROTOCOL

EXPRESSION BY INFECTION WITH M13 PHAGE mGP1-2

Whenever the gene for T7 RNA polymerase is present in *E. coli* cells, low levels of T7 RNA polymerase are constitutively produced. This can be a problem when the gene products under the control of p_{T7} are toxic. One strategy to avoid this is to keep the gene for T7 RNA polymerase out of the cell until the time of induction. In the protocol presented here, T7 RNA polymerase is introduced into the cell by infection with the M13 phage mGP1-2. This phage contains the gene for T7 RNA polymerase under the control of the *lac* promoter (Fig. 16.2.3). Host cells for this phage must carry the F factor so that they are susceptible to M13 infection (e.g., JM101 or K38). The cells are transformed with the single plasmid that contains the gene to be expressed under the control of p_{T7}. The cells are grown at 37°C, and induction occurs by infection with a high multiplicity of mGP1-2 in the presence of IPTG. A λ vector, CE6, that contains the gene for T7 RNA polymerase has also been used to express toxic genes (Studier and Moffatt, 1986; Studier et al., 1990).

Additional Materials

M13 phage mGP1-2 (available from author)
PEG solution (*UNIT 1.7*)
100 mM IPTG (Table 1.4.2)

Additional reagents and equipment for preparing M13 phage (*UNIT 1.15*) and titering phage (*UNIT 1.11*).

1. Prepare a stock of M13 phage mGP1-2 and concentrate the phage by precipitation with PEG solution. (DO NOT proceed to add TE buffer or phenol.) Resuspend phage in M9 medium and titer.

   If the cell proteins are to be labeled, it is important that the phage used to infect the cells are free of unlabeled methionine. In this case, precipitate the phage with PEG twice, each time resuspending the pellet in M9 medium. For long-term storage of the M13 phage mGP1-2, it is best to purify the phage through a CsCl gradient (Nakai and Richardson, 1986).

2. Transform *E. coli* cells susceptible to M13 infection (e.g., JM101 or K38) with the T7-promoter expression plasmid obtained from steps 1 and 2 of the basic protocol. Plate the transformants on LB/ampicillin plates and grow overnight at 37°C.

3. Pick a single colony and grow in LB/ampicillin medium overnight at 37°C.

4. Dilute the overnight culture of cells 1:100 in LB/ampicillin medium and grow several hours at 37°C with gentle shaking to $OD_{590} \cong 0.5$.

   It is very important that only gentle shaking is used when growing cells for M13 infection. Vigorous agitation results in shearing of the pili on the surface of the E. coli cells, resulting in inefficient infection.

5. Infect cells with M13 phage mGP1-2 (from step 1) at a ratio of ~10 phage for each *E. coli* cell. Add 100 mM IPTG to 1 mM final (a 1:100 dilution) to induce production

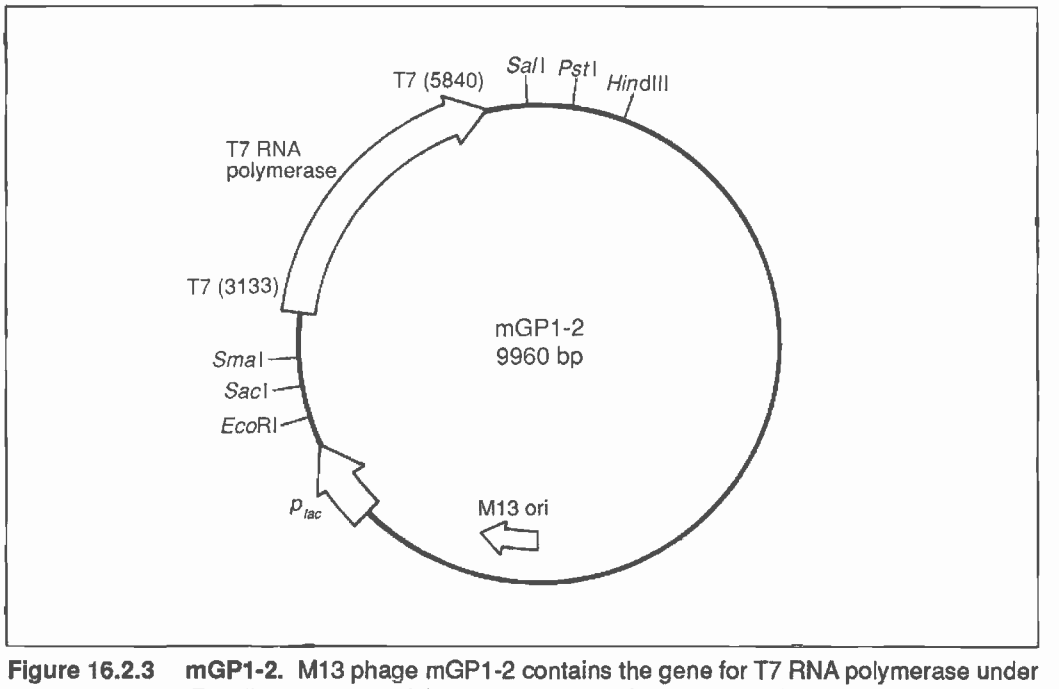

Figure 16.2.3 mGP1-2. M13 phage mGP1-2 contains the gene for T7 RNA polymerase under the control of the *E. coli* p_{lac} promoter. It is especially useful for the production of gene products that are toxic to the *E. coli* cell. When *E. coli* cells are infected with this phage, and IPTG is added to induce the p_{lac} promoter, T7 RNA polymerase is produced. As a result, any genes within the cell under the control of p_{T7} will be induced. mGP1-2 is described in Tabor and Richardson (1987).

of T7 RNA polymerase. Incubate the cells 2 hr at 37°C.

At $OD_{590} \cong 0.5$, the density of E. coli cells will be ~2×10^8 cells/ml. Thus, it is necessary to add M13 mGP1-2 phage at a final concentration of 2×10^9 phage/ml to obtain a multiplicity of infection of 10. Small cultures (~50 ml) can be incubated in a water bath without shaking. Larger cultures should be incubated at 37°C with gentle shaking.

6. Harvest cells and analyze induced proteins as in steps 9 and 10 of the basic protocol.

REAGENTS AND SOLUTIONS

18 amino acid mixture

Prepare a solution containing 0.1% (vol/vol) of each amino acid except cysteine (minus cysteine) and methionine (minus methionine). Filter sterilize through a 0.2-μm filter. Store at –20°C for several years.

Cracking buffer

60 mM Tris·Cl, pH 6.8
1% 2-mercaptoethanol
1% sodium dodecyl sulfate (SDS)
10% glycerol
0.01% Bromphenol Blue

COMMENTARY

Background Information

Bacteriophage T7 and T7-related phage (e.g., SP6, T3) encode their own RNA polymerase (see *UNIT 3.8*). Compared to other known RNA polymerases, this RNA polymerase is both relatively simple and highly efficient. T7 RNA polymerase is a single polypeptide of 96,000 kDa. It initiates transcription specifically at a 23-nucleotide promoter sequence, a sequence not present on the *E. coli* genome. Transcription is very processive, producing transcripts that are many thousands of nucleotides in length. Transcription is relatively rapid—five times the rate of *E. coli* RNA polymerase. All of these properties make T7 RNA polymerase and its promoter an attractive system for controlling the expression of foreign genes in *E. coli* and in other organisms. Expression systems in *E. coli* based on the controlled induction of T7 RNA polymerase have been developed by Tabor and Richardson (1985) and Studier and his colleagues (Studier and Moffatt, 1986; Rosenberg et al., 1987; Studier et al., 1990). The vectors described here are those developed by Tabor and Richardson. T7 RNA polymerase/promoter expression systems have also been successfully applied in yeast (Chen et al., 1987) and mammalian cells (Dunn et al., 1988; Fuerst et al., 1986).

Critical Parameters

Choice of vector

Questions that determine what vector to use to express a gene using T7 RNA polymerase include: Is there a ribosome-binding site upstream of the gene? What are the restriction sites available on each end of the gene? Is the gene product toxic to the *E. coli* cell? Examples of three standard vectors (pT7-5, pT7-6, and pT7-7) are shown in Figure 16.2.1. These vectors are derivatives of pBR322. The β-lactamase gene encoding amp^r is in the opposite orientation of p_{T7}; consequently the only plasmid-encoded genes expressed by T7 RNA polymerase are those cloned into the polylinker region. pT7-5 and pT7-6 contain the polylinker region located immediately downstream of p_{T7} in opposite orientations. There is no ribosome-binding sequence in these two plasmids, and thus they should be used either for the production of transcripts without expectation of good translation of the protein, or for the expression of genes that already have strong ribosome-binding sequences. pT7-7 differs from pT7-5 and pT7-6 in that it contains a strong ribosome-binding sequence between p_{T7} and the polylinker region; it is recommended for the expression of genes that lack a strong ribosome binding sequence or for the production of fusion proteins.

An extensive series of additional vectors containing p_{T7}, the pET series, have been described by Studier et al. (1990). These vectors are particularly useful for applications that require a greater selection of restriction endonuclease sites to insert the gene into, or that involve the expression of a gene that is toxic to the cell (see below). Some of these vectors contain other transcriptional regulatory elements (i.e., terminators, operators, RNase III cleavage sites) that could be of use for specific applications.

A large number of commercially available vectors contain a T7 RNA promoter (e.g., pIBI vectors, available from IBI; pSP6/T7-19, available from GIBCO/BRL; pBluescript II vectors, available from Stratagene; and pTZ18R and pTZ19R, available from U.S. Biochemical). These are intended to be used for producing specific transcripts in vitro using T7 RNA polymerase. In principle, they should be useful for the expression of genes using T7 RNA polymerase in vivo as well. In practice, however, the use of some of these vectors can result in some unexpected problems. (1) Most commercial vectors have extremely high copy numbers within the cell; this can accentuate the problems encountered with toxic genes. (2) In most vectors, the β-lactamase gene is oriented in the same direction as p_{T7}, complicating the analysis of radiolabeled proteins. (3) Some commercial vectors have p_{T7} oriented in a potentially deleterious direction. Derivatives of pBR322 that contain p_{T7} oriented clockwise with respect to the standard map are inviable in some *E. coli* strains that contain the gene for T7 RNA polymerase. This is due to the fact that high levels of transcription through the origin region of these plasmids in this orientation interferes with the replication of the plasmids. (4) Most commercial vectors have a *lac* operator sequence within them. This can titrate out the *lac* repressor (*UNIT 1.4*) and cause problems when the p_{lac} is used to control the T7 RNA polymerase gene.

Toxic genes

In some cases the gene to be expressed is toxic to the cells, even when it is not induced. This is due to a low level of constitutive expression present even under uninduced conditions. Although most genes are not toxic when expressed using the two-plasmid p_{T7} system, it is important to recognize the symptoms of toxicity to avoid selecting for mutations and to allow alternate systems for induction to be tried. The degree of toxicity varies greatly with each gene. The symptoms encountered with toxic genes are discussed below, in order of increasing toxicity.

Some genes are mildly toxic to the cells when expressed using the two-plasmid p_{T7} system. In such cases, the cells can be stably transformed with the two plasmids and the gene product is produced at a high level. However, after the cells are several days old, they no longer induce the expected gene product even though they remain resistant to ampicillin and kanamycin. To avoid this problem, it is recommended that the *E. coli* K38/pGP1-2 be stored in the absence of the plasmid containing p_{T7} as a glycerol stock at −80°C (*UNIT 1.3*). The plasmid containing p_{T7} and the gene to be expressed should be stored as DNA at −20°C or −70°C (*UNIT 1.6*). To prepare the strain for induction, streak K38/pGP1-2 on an LB/kanamycin plate at 30°C, grow up a single colony, transform with the plasmid containing p_{T7} and the gene to be expressed, and plate the transformants on LB/ampicillin/kanamycin plates at 30°C. A single colony should then be grown at 30°C and induced as described above. This procedure is not necessary for genes that are not toxic. Strains that do not induce toxic genes can be stored in glycerol at −80°C for many months (*UNIT 1.3*).

A more toxic class of genes consists of those that can be successfully cloned into a plasmid under the control of p_{T7}, but that render the resulting plasmid unable to stably transform a cell that contains the gene for T7 RNA polymerase. Genes that are toxic to the cells only in the presence of pGP1-2 (which expresses the T7 RNA polymerase) are relatively common, occurring on the average ~5% of the time (S. Tabor, unpublished observation). Note that such plasmids will give transformants in *E. coli* cells containing pGP1-2, but that the frequency of transformation will be greatly reduced (>50-fold) compared to the frequency of transformation by the parent vector alone. The cells that do grow in the presence of ampicillin and kanamycin will invariably contain deletions or other mutations in one of the two plasmids, and the desired gene product will not be produced.

When genes are toxic at this level, it is necessary to use an alternative strategy that reduces the expression of the gene under uninduced conditions. One strategy is to remove the gene for T7 RNA polymerase from the cell until induction is desired, and then introduce it by a phage infection. Such an alternate protocol is described using an M13 phage harbor-

ing the gene for T7 polymerase, mGP1-2. A lambda vector, CE6, that contains the gene for T7 RNA polymerase has also been used for this purpose (Studier and Moffatt, 1986; Studier et al., 1990). Another strategy is to retain the gene for T7 RNA polymerase in the cell but reduce the level of transcription by T7 RNA polymerase under uninduced conditions. For example, a system has been developed that expresses an inhibitor of T7 RNA polymerase—the T7 lysozyme—to reduce the activity of T7 RNA polymerase until it is induced (Studier et al., 1990). Another recent modification is the placement of p_{T7} under the control of the *lac* repressor, reducing the activity of T7 RNA polymerase until IPTG has been added (Studier et al., 1990).

Finally, some genes are difficult to clone in multicopy plasmids even in the absence of a known *E. coli* promoter. The difficulty in cloning these genes arises from the fact that their products are extremely toxic and that the residual low level of transcription by *E. coli* RNA polymerase in most plasmids is sufficient to direct the synthesis of small amounts of these proteins. One strategy that can be used to clone such toxic genes is to insert the gene near a strong *E. coli* promoter that is oriented so that transcription by the *E. coli* RNA polymerase results in the accumulation of RNA that is antisense to the toxic gene, reducing the level of its gene product. It is important to remember that the amount of a gene product synthesized is a function not only of the level of transcription but also of the efficiency at which translation is initiated. This is determined primarily by the ribosome-binding sequence located upstream of the start codon. Thus, some toxic genes with relatively weak ribosome-binding sequences can be cloned into multicopy plasmids, but not into a multicopy plasmid that also introduces a strong ribosome-binding sequence (S. Tabor, unpublished observations).

In summary, the first step in using the T7 RNA polymerase/promoter system is to clone the gene into an appropriate vector containing a p_{T7} and be certain it has an efficient ribosome-binding sequence. Once this is accomplished, the next step is determining whether the plasmid can stably transform an *E. coli* cell containing pGP1-2 at an efficiency comparable to that of the parent vector alone. If this is successful, the system is ready to be induced. If unsuccessful, it is necessary to induce the gene either by infection with M13 phage mGP1-2 (see second alternate protocol), or to use one of the more specialized vectors that further reduce the expression of T7 RNA polymerase in the cell under uninduced conditions (Studier et al., 1990).

Troubleshooting

For gene expression, one of the major advantages of the T7 RNA polymerase/promoter system over an *E. coli* RNA polymerase system is the ability to exclusively label the gene products under the control of p_{T7}. If the level of induction of the gene is estimated by inspection of a standard SDS-polyacrylamide gel, and it is difficult to see the expected induced product, then it is recommended that the induced proteins be labeled using [^{35}S]methionine as described in the first alternate protocol. This is a much more sensitive and specific assay for the specific protein production. Be sure that there is at least one methionine codon in the gene other than the one at the start of the protein (which is often removed in *E. coli*; Kirel et al., 1989); if not, then it is necessary to label with a cysteine or some other amino acid.

If it is not possible to detect the expected labeled product, there may be a problem with one of the two plasmids in the cell. One possibility is that the expressed protein is toxic to the cell, and as a result, a mutation has been selected for such that the toxic product is not synthesized. For more information on determining whether a gene is toxic, see the discussion on toxic genes in critical parameters. To determine if the cells and T7 RNA polymerase gene (e.g., pGP1-2) are inducing T7 RNA polymerase, attempt to induce a control protein that has been shown to work well in this system (e.g., the β-lactamase gene in pT7-1; Tabor and Richardson, 1985).

If the expressed protein does not accumulate significantly after induction, determine its stability in *E. coli* cells by pulse labeling with [^{35}S]methionine and chasing for various time periods with unlabeled methionine. If it is rapidly degraded, try to induce the gene in a protease-deficient strain. It should be noted that there are no known mutations that inactivate several very active *E. coli* proteases, and thus there is a strong probability that the mutant strains available (e.g., *lon*$^-$) will have no effect on the stability of the gene product. In addition, such mutant strains generally grow poorly, and as a consequence the gene products are poorly produced upon induction of T7 RNA polymerase.

The most common reason for poor induc-

tion of a gene is that the translation does not initiate efficiently. Therefore, it is very important that there be an efficient ribosome-binding sequence the proper distance upstream of the gene. If a gene product does not induce well, and the problem is not the stability of the product, try a different ribosome-binding sequence—one that is known to work efficiently. The sequence and spacing between the ribosome-binding sequence and the start codon is critical. Because of this, it is recommended that the gene be inserted into a vector such as pT7-7, without altering any of the sequences between the ribosome-binding sequence and the start codon.

Anticipated Results

Under optimal conditions, the gene product expressed by the T7 RNA polymerase/promoter system can accumulate to >25% of the total cellular protein. However, in most instances the amount of gene product that accumulates is significantly less than this. There are numerous reasons for poor yields of gene product, as discussed in troubleshooting (see above).

Time Considerations

It should take ~1 week to insert the gene of interest into the p_{T7} vector, prepare minipreps of the DNA, and characterize the recombinants for the correct size and orientation of the insert. It should then take 3 days to transform the recombinant plasmid into the *E. coli* strain containing pGP1-2, induce the cells, and test the extracts for the production of the expected gene product.

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Key References

Studier et al., 1990. See above.

Gives extensive list of vectors and protocols for expression using T7 RNA polymerase.

Tabor and Richardson, 1985. See above.

Describes the use of the two-plasmid system for expression of genes using T7 RNA polymerase.

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Expression Using Vectors with Phage λ Regulatory Sequences

Many expression systems have been developed that utilize pBR322-based plasmids into which transcriptional and translational regulatory signals have been inserted. In the system described here, however, plasmids (pSKF) utilize regulatory signals—such as the powerful promoter p_L—from the bacteriophage λ. Transcription from p_L can be fully repressed and plasmids containing it are thus stabilized by the λ repressor, *c*I. The repressor is supplied by an *E. coli* host which contains an integrated copy of a portion of the λ genome. This so-called defective lysogen supplies the λ regulatory proteins *c*I and N but does not provide the lytic components that would normally lead to cell lysis. Thus, cells carrying these plasmids can be grown initially to high density without expression of the cloned gene and subsequently induced to synthesize the product upon inactivation of the repressor.

This system also ensures that p_L-directed transcription efficiently traverses any gene insert, which is accomplished by providing the phage λ antitermination function, N, to the cell and by including on the p_L transcription unit a site necessary for N utilization (Nut site). The N protein interacts with and modifies the RNA polymerase at the Nut site so as to block transcription termination at distal sites in the transcription unit.

In order to express the coding sequence, efficient ribosome-recognition and translation-initiation sites have been engineered into the p_L transcription unit. Expression occurs after temperature or chemical induction inactivates the repressor (see basic protocols). Restriction endonuclease sites for insertion of the desired gene have been introduced both upstream and downstream from an ATG initiation codon. Thus, the system allows either direct expression or indirect expression (via protein fusion) of any coding sequence, thereby potentially allowing expression of any gene insert. Direct expression generates "authentic" gene products (first support protocol), while expression of heterologous genes fused to highly expressed gene partners generates chimeric proteins that differ from the native form. In the latter case, the fusion partner can be removed to obtain an unfused version of the gene product (second support protocol).

BASIC PROTOCOL

TEMPERATURE INDUCTION OF GENE EXPRESSION

Expression from p_L-containing vectors can be induced by raising the temperature. The *E. coli* lysogens used with these vectors are typically defective for phage replication and carry a temperature-sensitive mutation in the phage λ *c*I gene (*c*I857). After transformation and growth, induction is accomplished by raising the temperature of the culture from 32° to 42°C.

Materials

Expression vector (e.g., pSKF series; see support protocols)
E. coli AR58 or equivalent (Table 1.4.5)
LB plates containing the appropriate antibiotic (*UNIT 1.1*)
LB medium containing appropriate antibiotic (room temperature and prewarmed to 65°C; *UNIT 1.1*)
SDS/sample buffer (*UNIT 10.2*)
Gyrotory air or water shaker, 32° and 42°C
Additional reagents and equipment for transformation (*UNIT 1.8*)

1. Transform the expression vector into an *E. coli* λ lysogen (such as AR58) carrying a temperature-sensitive mutation in its repressor gene (λ *c*I857). Plate on LB/antibiotic plates and incubate transformants at 32°C.

Heat-shock at 37° or 42°C for ≤90 sec during transformation is not a problem.

2. Grow the transformed cells overnight at 32°C in LB/antibiotic medium.
3. Dilute the overnight culture ≥1:20 into fresh LB/antibiotic medium. Grow the culture at 32°C in a gyrotory shaker at 250 to 300 rpm until OD_{650} = 0.6 to 0.8.
4. Add 1/3 vol of 65°C LB/antibiotic medium with swirling in order to elevate the culture temperature rapidly to 42°C.

   In our experience, a rapid increase in temperature favors production. Small shake-flask cultures (≤25 ml) are more easily induced by transfer to a 42°C gyrotory water bath without addition of prewarmed media. This generally raises the culture temperature to 42°C within 3 to 5 min.

5. Continue growing the culture 2 to 3 hr at 42°C.
6. Remove a 1-ml aliquot for analysis and harvest the remainder of cells by centrifuging 15 min in a low-speed rotor at 3000 × *g*, 4°C. Discard the supernatant.

   Freeze cell pellet at –70°C until ready to isolate the gene product.

7. Spin the 1-ml aliquot 1 min at top speed in a microcentrifuge, then resuspend the pellet in 50 µl SDS/sample buffer. Boil 5 to 10 min and analyze gene product by SDS–polyacrylamide gel electrophoresis.

CHEMICAL INDUCTION OF GENE EXPRESSION

BASIC PROTOCOL

Expression using the pSKF system can also be induced chemically in lysogens that carry a wild-type (*ind*$^+$) repressor gene (*c*I857 cannot be used as it is *ind*$^-$). This is accomplished by treating the bacterial host with an agent such as nalidixic acid. Nalidixic acid inhibits DNA gyrase and leads to DNA damage, which induces the SOS response. During the SOS response, wild-type repressor protein is cleaved. In this case, the wild-type repressor protein is cleaved by the RecA protease, which is induced by the SOS response. In contrast to induction by heat (product accumulates in 45 to 90 min) nalidixic acid–mediated induction of protein expression is comparatively slow (product accumulates in 5 to 6 hr).

Materials

Expression vector (e.g., pSKF series; see support protocols)
E. coli AR120 or equivalent (Table 1.4.5)
LB plates containing appropriate antibiotic (*UNIT 1.1*)
LB medium containing appropriate antibiotic (*UNIT 1.1*)
60 mg/ml nalidixic acid in 1 N NaOH (not necessary to filter sterilize; Table 1.4.1)
Additional reagents and equipment for transformation (*UNIT 1.8*)

1. Transform the expression vector into a replication-defective, *E. coli* *c*I$^+$ lysogen (e.g., AR120). Plate on LB/antibiotic plates and incubate the transformants at 37°C.
2. Grow the transformed cells overnight at 37°C in LB/antibiotic medium.
3. Dilute the overnight culture ≥1:20 into fresh LB/antibiotic medium. Grow the culture at 37°C in a gyrotory shaker at 250 to 300 rpm until OD_{650} = 0.4.
4. Add 1/1000 vol of 60 mg/ml nalidixic acid solution to give 60 µg/ml final concentration.
5. Continue growing the culture 5 to 6 hr at 37°C.
6. Harvest cells and analyze gene product (steps 6 and 7 of first basic protocol).

SUPPORT PROTOCOL

AUTHENTIC GENE CLONING USING pSKF VECTORS

It is often most desirable to express a gene product in a form as similar to the native protein as possible. Such an "authentic" gene product will have the greatest chance of having a structure and activity identical to that of the native protein. Efficient translation of a coding sequence for an authentic gene product is typically accomplished by placing the inserted information immediately adjacent to a ribosome-binding site (a translational regulatory signal that interacts with the 16S rRNA of *E. coli* and contains an ATG initiation codon; Gold et al., 1981).

Strategic Planning

The translation-initiation signal utilized here is that of the phage λ *c*II gene. In order to make the translational information generally useful, the coding region of the gene has been removed from the vectors, leaving only their initiator *f*Met codon and upstream translational regulatory sequences. Additionally, these vectors have been engineered to provide restriction endonuclease sites on either side of the ATG, such that the initiation codon can be supplied by either the plasmid or the gene being inserted. Finally, restriction sites have also been engineered upstream of the translational regulatory region to permit insertion of other ribosome-binding sites. Those genes that contain restriction sites compatible with the sites on the vector may be inserted directly into the vector. As most genes do not contain appropriately positioned restriction sites, it is often necessary to adapt existing restriction cloning sites within the gene to fuse it to the translation-initiation signals provided by the vectors.

For example, pSKF101 (Fig. 16.3.1) and pSKF102 both have a *Bam*HI site adjacent to the initiation codon (ATGgatcc), while pSKF201 has an *Nco*I site (ccATGg) and pSKF301 (Fig. 16.3.2) has an *Nde*I site (catATG). The protocol presented below summarizes the steps to obtain an authentic gene clone using pSKF101 as an example.

Sample Protocol

Materials

Appropriate restriction endonucleases and buffers (*UNIT 3.1*)
pSKF101 vector (available from A. Shatzman; Fig. 16.3.1)
Competent *E. coli* AS1 (Table 1.4.5; also known as MM294*c*I$^+$)

Additional reagents and equipment for restriction digestion, (*UNIT 3.1*), oligonucleotide synthesis and purification (*UNITS 2.11 & 2.12*), nondenaturing PAGE (*UNIT 2.7*), isolation, recovery, and quantitation of DNA (*UNIT 2.6 & APPENDIX 3*), subcloning DNA fragments (*UNIT 3.16*), transforming, plating, and growing *E. coli* (*UNITS 1.8, 1.1, & 1.3*), and DNA miniprep (*UNIT 1.6*)

1. Identify a unique restriction endonuclease site close to the 5′ end of the coding sequence of the gene to be expressed, as well as another unique site 3′ to this gene's termination codon.

2. Synthesize two single-stranded DNA oligonucleotides, recreating the coding sequence immediately preceding the unique restriction endonuclease site near the 5′ end of the gene to be expressed. Purify and quantitate the DNA, then anneal in order to obtain double-stranded DNA.

   This synthetic DNA sequence is used to link the gene to be expressed to the initiating ATG of the pSKF expression vector. The double-stranded oligonucleotide should be designed to have ends that are complimentary to the restriction sites identified at the 5′ end of the gene to be expressed as well as the chosen restriction site in the expression vector.

3. Digest 25 to 50 µg plasmid DNA containing the gene to be expressed with the restriction endonucleases identified in step 1.

   To ensure complete digestion, determine that the restriction endonuclease buffer is appropriate for each enzyme to be used. If the endonucleases require different buffers, then each restriction digestion must be done separately.

4. Electrophorese the doubly digested plasmid DNA on a polyacrylamide gel.

   If the DNA fragment to be isolated is between 150 and 1100 bp, a 6% gel can be used. Either a borate- or acetate-buffer system can be used. If digestion was done in a large volume, ethanol precipitate the DNA (UNIT 2.1) and resuspend in 40 to 100 µl TE buffer. Mix with loading dye and load.

5. Locate the fragment of interest by staining with an agent such as ethidium bromide and cut the DNA fragment out of the gel.

6. Recover the DNA by electroelution and quantitate the amount of DNA.

   Confirm that the correct fragment has been isolated by running a small aliquot on an agarose gel. Be sure to run appropriate size markers in an adjacent lane.

7. Digest 10 µg pSKF101 with *Bam*HI and a restriction endonuclease that generates ends compatible with the 3′ end of the coding sequence (to accommodate the 3′ end of the gene to be expressed).

   Confirm that complete digestion of the vector has occurred by analysis of digested

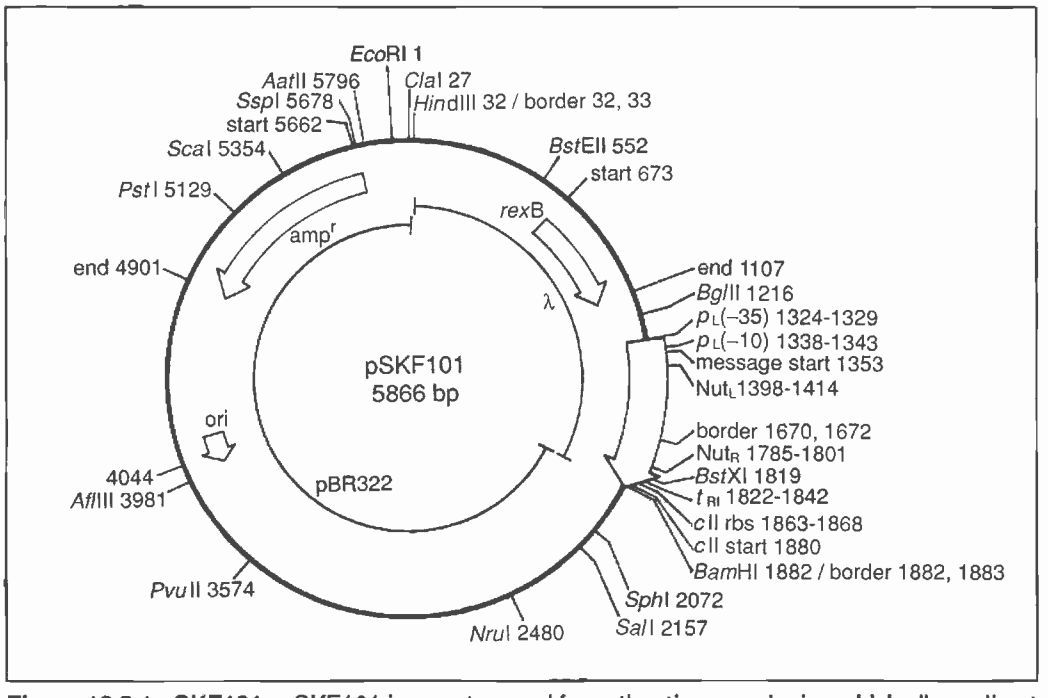


Figure 16.3.1 pSKF101. pSKF101 is a vector used for authentic gene cloning which allows direct expression of the inserted gene. It is a derivative of pBR322 (UNIT 1.5) containing sequences inserted between *Hin*dIII and *Bam*HI sites of pBR322. The inserted λ sequences contain the p_L promoter and *c*II ribosome-binding site (rbs); these are the transcriptional and translational regulatory sequences necessary to express heterologous genes in *E. coli*. Within this region are several unique restriction sites that permit insertion of the gene. The regions derived from pBR322 and λ are indicated. This plasmid can be maintained stably in a λ-lysogenized *E. coli* strain. The selectable marker is ampicillin, encoded by β-lactamase.

An alternative name for pSKF101 is pASI (Rosenberg et al., 1983). Alternative names of related vectors are as follows: pSKF102 is pOTSV (Shatzman and Rosenberg, 1987); pSKF201 is pOTS-Nco (Shatzman and Rosenberg, 1987); and pSKF301 is pMG1.

DNA on an agarose gel. Compare undigested pSKF101 with digested to make sure that pSKF101 has been completely linearized.

8. Prepare a ligation reaction (using the conditions described in *UNIT 3.16*) by combining the following ingredients:

   1 ng digested pSKF101 vector DNA
   10 ng of the gene fragment to be expressed (from step 6)
   20 ng synthetic oligonucleotide (from step 2)
   T4 DNA ligase.

   Ligate 10 to 12 hr at 4°C.

   There is no need to dephosphorylate pSKF101 as long as there is at least a 5-fold molar excess of vector DNA to isolated DNA fragment and synthetic DNA.

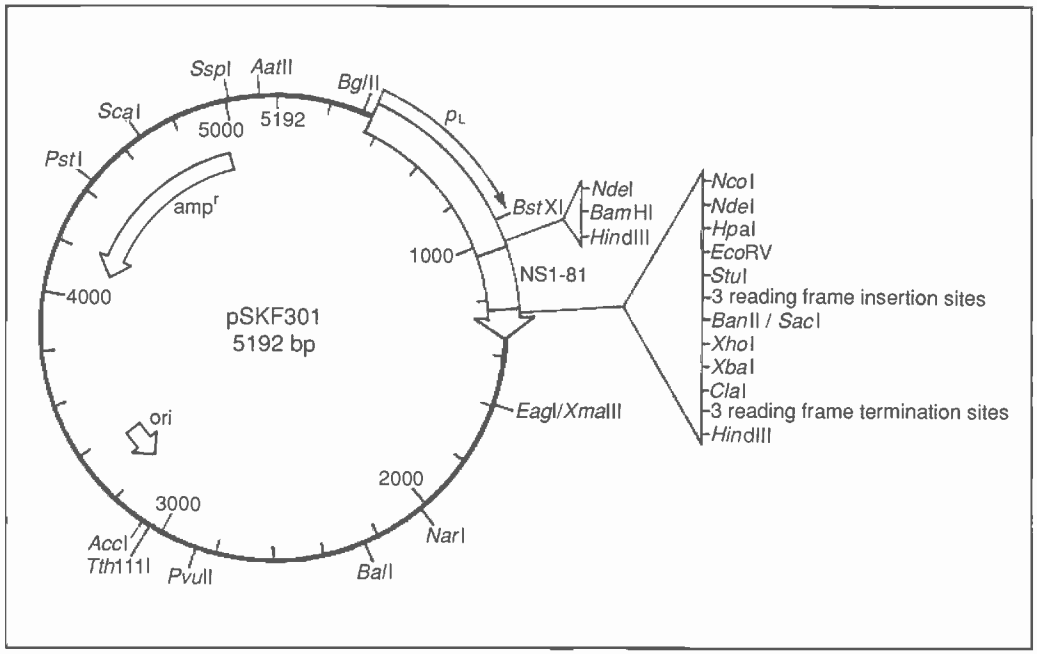
9. Remove one-third of the ligation reaction and transform 50 to 100 µl competent *E. coli* AS1. Plate on LB/ampicillin plates and incubate overnight at 37°C.

10. Pick 12 to 24 colonies and transfer with a sterile toothpick to 3 ml LB/ampicillin medium. Grow cells 5 to 18 hr and isolate DNA by a miniprep method.

    Cells may be harvested once the broth appears turbid. For best results, allow 8 to 12 hr of growth.

11. Perform appropriate restriction endonuclease digests to determine which clones contain the desired construction of the gene to be expressed.

12. Transform an *E. coli* strain with the DNA and express the gene as in the basic protocols.



16.3.2 pSKF301. pSKF301 is a vector that can be used for both indirect and direct expression. It is similar to pSKF101 in that it contains the same transcriptional and translational regulatory sequences as well as selectable markers; it differs in that it contains a shorter segment of λ DNA than pSKF101. pSKF301 also contains the coding sequence of the first 81 amino acids of the influenza protein, NS1, shown as NS1-81. This region is adjacent to the cII ribosome-binding site (rbs) and contains restriction sites at the 3′ end of NS1-81 that allow construction of translational fusions in any of the three reading frames. Removal of NS1-81 permits direct expression of the cloned gene. (This vector is also known as pMG1.)

CONSTRUCTION AND DISASSEMBLY OF FUSED GENES IN pSKF301

By fusing the gene to be expressed to a coding region of another gene (the fusion partner), a chimeric gene can be constructed in an appropriate vector. Numerous vectors are available for this purpose but most share the common feature of a fusion partner that is a highly expressed gene. When expression of the chimeric gene is induced, the resulting proteins carry additional peptide information at the N terminus. Although the fusion product may have physical and/or functional properties that differ from the "authentic" protein, advantages of the approach include highly efficient expression (up to 30% of total cell protein) without complicated alterations on the gene, and the presence of a "handle" on the expressed protein which can help to identify and purify it. Such proteins are often used to develop antisera to specific proteins that have diagnostic potential, and have been used successfully to identify and define a variety of gene products (Casadaban et al., 1983; Rose and Botstein, 1983; Guarente, 1983).

Strategic Planning

Plasmid pSKF301 has been constructed to permit initial expression of a gene as a fusion product, followed by removal of the DNA encoding the fusion portion by restriction digestion. Finally, the unfused version of the gene is expressed as an authentic protein.

pSKF301 contains an *Nde*I restriction site adjacent to the ATG following the *c*II ribosome-binding site (Fig. 16.3.3). This ATG also serves as the translational start (Gold et al., 1981) of the *NS1* gene derived from the influenza nonstructural gene. This gene has been truncated to express only its first 81 amino acids. Just beyond the coding sequence for the 81st amino acid is a second *Nde*I site followed by three unique blunt-ended restriction sites, *Hpa*I, *Eco*RV, and *Stu*I, which allow for the insertion of genes into any of three reading frames. Immediately following the *Stu*I site are sequences coding for translational stops in any of the three reading frames.

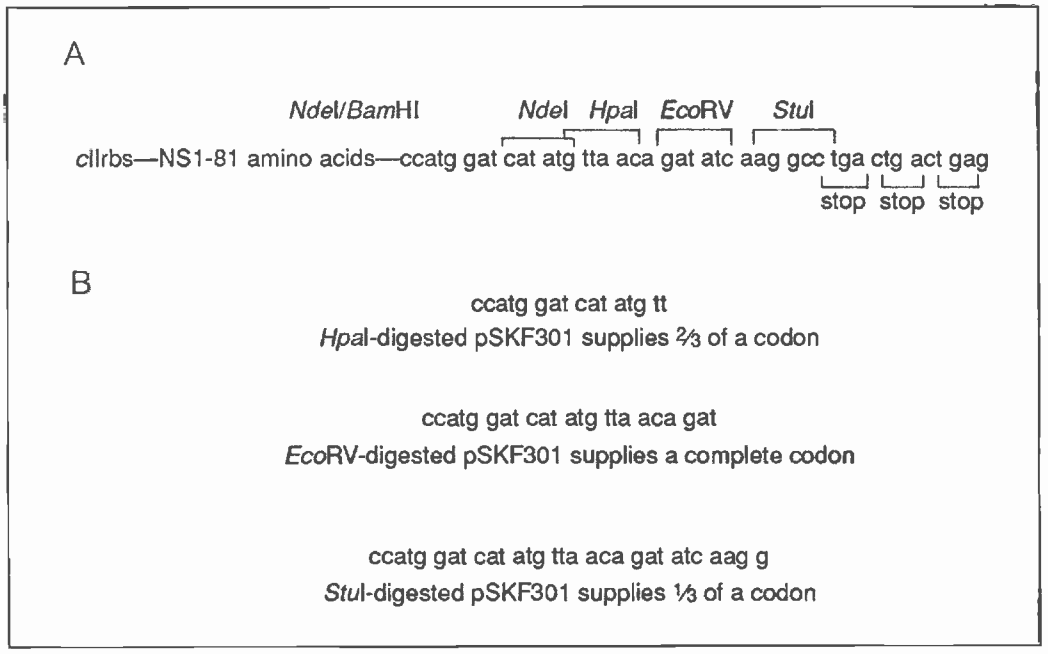


Figure 16.3.3 Sequence and restriction endonuclease sites (in the region used for cloning) of pSKF301 (**A**). Restriction endonuclease digestion shows the strategy utilized to obtain pSKF301 as a vehicle for expression in all three reading frames (**B**).

The expression of a gene of interest as a fusion protein may be achieved by utilizing any of the following restriction sites in pSKF301: *Nco*I, *Hpa*I, *Eco*RV, or *Stu*I. Choice of restriction site depends upon the reading frame necessary for the translation of a specific protein sequence. First, a unique restriction site close to the 5′ end of the gene (or portion of the gene) to be expressed must be identified. Second, the appropriate restriction endonuclease is selected for digesting pSKF301 such that the gene will be expressed.

If the chosen restriction site is a blunt-end cutter, no further manipulation of that end is required. In the event the restriction site identified leaves either a 5′ or 3′ protruding end, further manipulation is required. "Filling in" using the Klenow fragment of *E. coli* DNA polymerase for 5′ protrusions or T4 DNA polymerase, or S1 or mung bean nucleases for 3′ protrusions, are methods of choice (see *UNIT 3.16*).

Sample Protocol

Materials

Appropriate restriction endonucleases and buffers (*UNIT 3.1*)
Klenow fragment of *E. coli* DNA polymerase I (*UNIT 3.5*)
pSKF301 vector (available from A. Shatzman; Figs. 16.3.1 & 16.3.2)
T4 DNA ligase (*UNIT 3.14*)
Competent *E. coli* AS1 (Table 1.4.5; also known as MM294*c*I$^+$)
Additional reagents and equipment for large-scale plasmid prep (*UNIT 1.7*), agarose gel electrophoresis (*UNIT 2.5*), extraction and precipitation of DNA (*UNIT 2.1*), transformation of competent cells (*UNIT 1.8*), and restriction digestion and mapping (*UNITS 3.1-3.3*)

Construct a gene fusion in pSKF301

1. Assume the restriction site identified in the gene is a *Bam*HI site. Digest with *Bam*HI to obtain:

GATCC	XXX	XXX	XXX	XXX
G	YYY	YYY	YYY	YYY

2. Treat with Klenow fragment to fill in the unpaired bases to obtain:

GATCC	XXX	XXX	XXX	XXX
CTAGG	YYY	YYY	YYY	YYY

As noted above, Klenow fragment is used to fill in for 5′ protrusions. For 3′ protrusions, use T4 DNA polymerase (UNIT 3.5) or S1 or mung bean nuclease (UNIT 3.12).

3. Determine the proper reading frame of the gene. In this example assume XXX XXX XXX XXX is the proper reading frame; therefore, the coding sequence of the filled-in fragment should read:

GA	TCC	XXX	XXX	XXX	XXX

4. Determine which restriction endonuclease should be used to digest pSKF301 to allow expression of the fusion protein. For this example, *Stu*I is required to yield:

ccatg gat cat atg tta aca gat atc aag gGA TCC XXX XXX XXX XXX

pSKF301 fusion gene

5. Prepare the vector and the fragment of the gene to be expressed as in the first support protocol, steps 3 to 12 (*except* no synthetic DNA is required).

Generate an authentic version of the gene

Once a gene has been expressed as a fusion protein, it may be desirable to obtain an unfused version of the gene product. If this is useful, follow steps 6 to 12.

To convert a fusion protein to an unfused protein when using pSKF301, be certain that the gene of interest does not contain an *Nde*I site. The following theoretical fusion construct will be used as an example in these steps:

*Nde*I *Nde*I

CATATGGATCC---NS1-81---CCATGGATCATATGTT---fusion gene---tga

6. Set up a large-scale plasmid preparation of the fusion construct to yield ~100 µg plasmid DNA.
7. Digest 10 µg of the construct with *Nde*I. Verify that all of the vector DNA has been completely digested by taking a small aliquot of the digested material and running it on an agarose gel next to lanes containing uncut plasmid and appropriate size markers. A 280-bp fragment should be observed; this contains the NS1-81 gene sequence being liberated from the construct.

   Confirmation of complete digestion is extremely important.
8. Purify the digested construct by phenol/chloroform/isoamyl alcohol extraction followed by ethanol precipitation.
9. Add T4 DNA ligase to 1 µg of the *Nde*I-digested construct and incubate overnight at 4°C.
10. Transform ligated DNA into competent *E. coli* AS1 cells (or any other suitable cI^+ lysogen).
11. Determine that the construct no longer contains the NS1-81 gene sequence by restriction analysis.

    Consult the restriction map of pSKF301 and the gene to be expressed to determine which endonucleases are diagnostic for identifying the construct devoid of the NS1-81 gene. If the NdeI digestion was complete upon ligation, reclosure is highly efficient. Expect 95% to 100% of the resulting transformants to contain the unfused construct.
12. Transform the DNA and express the gene by temperature or chemical induction as in the basic protocols.

COMMENTARY

Background Information

Expression of a heterologous gene or gene fragment in *E. coli* requires that the coding sequence be placed under the transcriptional and translational control of regulatory elements recognized by the bacterial cell. The pSKF vectors were designed specifically to direct gene expression by providing regulatory signals from bacteriophage λ. Phage regulatory signals were chosen because of their high efficiency and ability to be tightly regulated. This system uses a promoter that can be tightly controlled, eliminating problems with "leaky" basal expression sometimes found in other expression systems (see below). This system uses an antitermination mechanism to help assure efficient transcription across any gene insert. The different vectors used with this system offer several choices of antibiotic selection markers, contain elements that optimize plasmid stability, and carry a variety of restriction sites that permit relatively easy insertion of the gene of interest adjacent to the efficient translation regulatory information.

The pSKF system offers some advantages that differentiate it from many other expres-

sion systems. Perhaps most important is the "tightness" of regulation of the p_L promoter. Several other strong regulatable promoters—p_{tac} (de Boer et al., 1982), p_{trp} (Edman et al., 1981), and p_{T7} (promoter of T7 gene 10; Studier and Moffatt, 1986)—are also used routinely for optimizing heterologous gene expression in *E. coli*. These promoters, along with p_L, are all of comparable strength and are sufficient to achieve very high levels of mRNA production (*UNIT 16.1*). In fact, these promoters are so powerful that further enhancement of promoter strength would not be expected to result in an increase of protein production; indeed, these promoters are so strong that it is very difficult to keep them fully turned off even in the "repressed" state. Because of this basal transcription under repressed conditions, use of the p_{tac}, p_{trp}, or p_{T7} (coupled with the p_{lac}-T7 polymerase) systems often leads to some expression of the cloned gene even under nonpermissive conditions. This may lead to plasmid loss or rearrangement, or possibly cell death, if small amounts of the gene product are lethal to *E. coli*. In contrast, one does not typically see expression of the cloned gene in the p_L system until cultures have been induced.

A second advantage of the p_L system over other promoter systems is the flexibility gained from having completely different induction systems (thermal and chemical). In contrast, the p_{tac} and p_{T7} systems mentioned above permit induction only by a chemical route. Different routes of induction lead to completely different cellular states (e.g., different physiology, morphology, and growth patterns) and these variations can lead to significant differences in gene product accumulation and stability (unpub. observ.). It should be pointed out that p_{trp} also permits dual modes of induction (by β-indolyl acetic acid or Trp starvation) and that a different version of the p_{T7} system has been developed (*UNIT 16.2*; Tabor and Richardson, 1985) in which the T7 RNA polymerase is thermally regulated via the p_L-*c*I857 system (however, this system is not chemically inducible as well).

The third major advantage of the pSKF system is the availability of a single vector that permits expression of either an authentic or a fusion gene product; furthermore, this vector allows the fusion gene to be converted to an authentic gene by a simple restriction digest followed by self ligation. Thus, a gene may be rapidly expressed at high levels as a protein fusion to give an initial reagent for use in activity studies and antisera preparation. Time may then be taken to optimize the expression of the authentic (nonfusion) gene product, which will be better suited for functional and structural studies. Most other expression systems do not provide this flexibility.

Critical Parameters

Gene expression is not solely a function of message levels. The efficiency of the ribosome-binding region—including the sequences both upstream and downstream of the ATG initiation codon—also play a role in determining the extent to which a protein is made. Alterations in these sequences may affect the secondary structure of a message and the conformational presentation of the initiation signals which, as a result, can alter translational efficiency (Gold et al., 1981).

From our experience, the host strain plays a major role in determining the ultimate levels of gene expression. The reasons for the rather dramatic differences seen in product yield from different host strains are poorly understood. Product stability is, however, one determining factor that has been somewhat characterized. Host strains have been developed that are defective in certain proteases (*UNIT 16.6*). These specialized host strains can have a significant impact on the expression of certain gene products. However, proteases are not the only factor involved in strain-to-strain variations observed in protein expression. Other uncharacterized factors can have equally dramatic effects. It is therefore recommended that expression be tried in a number of different *E. coli* strains.

Following the induction of cultures carrying the desired expression vector, cells may be analyzed in a variety of ways to detect the presence of the cloned gene product. Most typically, the presence of the novel gene product is determined directly by observing in SDS–polyacrylamide gels a new, inducible protein band not present in lanes from control cultures.

The expression of any gene insert can also be identified and/or confirmed in several ways related to the activity or function of the protein including: (1) direct detection of a novel function or activity imparted to the living bacterial host; (2) genetic complementation of the appropriate mutant host; (3) assay of whole-cell extracts for the activity of the cloned gene product; and (4) assay after partial or complete purification of the cloned gene product.

Immunochemical methods such as immunoprecipitation (*UNIT 10.16*) or western blotting (*UNIT 10.8*) are some of the most sensitive meth-

ods available to detect expression of a gene product. These methods, of course, require that an antiserum be available which is specific for the protein to be expressed. These methods, however, are primarily quantitative and do not necessarily indicate anything about the level of expression, homogeneity, or activity of the gene product.

If a good antiserum to the protein of interest is not available, purification of sufficient amounts of a gene product allows generation of high-titer, antigen-specific mono- or polyclonal antisera (*UNITS 11.3-11.13*). One approach to generate an antiserum is to produce the desired heterologous gene product in bacteria as a native protein, as a fusion, or as a protein fragment. The protein may then be purified and used to produce high-titer mono- or polyclonal antisera. Such antisera have been used to (1) map natural expression of the gene product with respect to cell type, subcellular distribution, and temporal regulation; (2) determine relative levels of expression in various cell types; (3) study protein processing and stability; (4) map immuno-dominant domains; (5) purify by immunoaffinity both the native and modified forms of the protein; and (6) provide in vivo diagnostic reagents for examining tissue distribution and expression of the gene product by immunofluorescent methods.

Troubleshooting

There is never a guarantee that a gene will be expressed at high levels, but poor expression upon initial trials does not signify defeat. As mentioned earlier, transcription is rarely limiting and is, therefore, not the first parameter to be addressed in attempting to improve expression. Instead, the easiest parameter to change is the host strain being used for production. Typically, five or six different strains (which might or might not be closely related to each other) may have to be tested in pilot experiments to see which gives optimal production.

The next parameter to examine in the event of poor expression is translation. Expression may be increased by altering ribosome-binding sites to improve complimentarity to the 16S rRNA, or by increasing the A-T richness of the 5′-end of the gene's coding region.

After steps have been taken to optimize translation, it is often helpful to alter the promoter and repressor system in order to change the induction system and the physiology of the cells during the production phase. For example, inducing the *c*I857-containing p_L system via a temperature shift generates a cellular heat-shock response and protein synthesis at 42°C. Induction of this system with nalidixic acid leads to a cellular SOS response (see glossary, *UNIT 1.0*) and protein production at 37°C. Induction of the *trp* system by tryptophan starvation turns on the host stringent response (a generalized response of *E. coli* to amino acid starvation). Thus, in each case, a different host response leads to induction of a different set of host proteins as well as to greatly different physiological effects (such as changes in respiration, filamentation, and growth rate).

Finally, it may be possible to improve expression by optimizing the temperature at which the protein is made, as this parameter has often been shown to affect the proteins' solubility, stability, and activity.

Anticipated Results

Expression of most gene products as fusions with the first 81 amino acids of the NS1 protein (using pSKF301) can be achieved at levels between 5% and 30% of total cellular protein. Expression levels of nonfusion proteins (authentic) are less predictable and may vary from <1% to 30% of total cell protein. By systematically optimizing each of the parameters described in the troubleshooting section, it may be possible to increase the level of expression of a nonfusion gene product from the low end of this range to the high end. In shake flask cultures this is equivalent to hundreds of milligrams per liter and in fermenters (where greater cell densities are achieved) to grams per liter of the desired product. The expressed product can usually be visualized by running a small aliquot of cell extract on an SDS-polyacrylamide gel and staining with Coomassie Brilliant Blue. The majority of the protein produced will be insoluble, but can often be solubilized and renatured to an active state (*UNIT 16.5*).

Time Considerations

Cell growth, induction, and harvesting require 6 to 8 hr depending on the strain of *E. coli* and the mode of induction used. Following harvest, the cell pellets may be frozen at −70°C for long periods with no obvious loss of gene product. It is often convenient to analyze a small aliquot of the induced culture (removed prior to harvesting the remainder of the culture) by SDS-PAGE the next day, as this step will require several hours including gel preparation, gel running, staining, and destaining. Once it is clear that sufficient levels of protein

have been produced to merit purification efforts, cells may be lysed and product extracted (if insoluble) by use of detergents and/or chaotropes. This will take 1 to 2 days depending on the number of extraction steps required and the length of dialysis steps chosen between extractions.

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Introduction to Expression by Fusion Protein Vectors

Expression—the directed synthesis of a foreign gene—is often the logical next step for researchers who have isolated a gene and want to study the protein it encodes. During the early days of recombinant DNA technology, it was thought that a strong promoter and a start codon at the beginning of the gene would be sufficient for good expression in *Escherichia coli*. Since then it has been learned that the requirements for efficient translation are a good deal more complicated. In addition to a promoter and a start codon, good expression requires that the mRNA encoding the protein to be expressed contain a ribosome-binding site that is not blocked by mRNA secondary structure. The level of expression is also affected by codon preferences, especially in the second codon of the gene (Stormo et al., 1982), and may be affected by the coding sequence in other ways that are not yet well understood (*UNIT 16.1*). In virtually all cases, these problems can be solved by altering the sequence preceding the start codon, and/or by making changes in the 5′ end of the coding sequence that do not change the protein sequence, taking advantage of the degeneracy of the genetic code.

However, it is often quicker to solve these problems by making fusions between genes. In this approach the cloned gene is introduced into an expression vector 3′ to a sequence (carrier sequence) coding for the amino terminus of a highly expressed protein (carrier protein). The carrier sequence is often from an *E. coli* gene, but it can be from any gene that is strongly expressed in *E. coli*. The carrier sequence provides the necessary signals for good expression, and the expressed fusion protein contains an N-terminal region encoded by the carrier. In such vectors, the portion of the fusion protein encoded by the carrier can be as small as one amino acid (*UNIT 16.3*; Amann and Brosius, 1985), although expression from such vectors can still be subject to problems caused by the coding sequence of the expressed protein. Perhaps more typical examples of short carrier sequences are those contained in the *trpE* vectors (*UNIT 16.5*) or the λ cII vectors (Nagai and Thøgersen, 1987).

The carrier sequence can also code for an entire functional moiety or even for an entire protein. For example, the following four units (*UNITS 16.5-16.8*) describe the use of vectors that express β-galactosidase and *trpE* fusions, maltose-binding protein (MBP) fusions, glutathione-S-transferase (GST) fusions, and thioredoxin (Trx) fusions. These carrier regions often can be exploited in purifying the protein, either with antibodies or with an affinity purification specific for that carrier protein. Alternatively, unique physical properties of the carrier protein (e.g., heat stability) can be exploited to allow selective purification of the fusion protein. In addition, some carrier proteins such as MBP and Trx can be selectively released from intact cells by osmotic shock or freeze/thaw procedures, even though they reside in different cellular compartments. Often, proteins fused to these carriers can be separated from the bulk of intracellular contaminants by taking advantage of this attribute.

There are three problems often encountered when expressing fusion proteins: solubility of the expressed protein, stability of the expressed protein, and presence of the carrier protein. The first two problems are often encountered with both fusion and nonfusion expression systems (*UNIT 16.1*), while the third is unique to fusion systems.

SOLUBILITY OF THE EXPRESSED PROTEIN

The high-level expression of many proteins can lead to the formation of *inclusion bodies*, very dense aggregates of insoluble protein and RNA that contain most of the expressed protein (Schein, 1989). Precipitation of a protein into inclusion bodies sometimes can work to one's advantage, because inclusion bodies are insoluble and dense, and can be purified relatively easily by centrifugation (*UNIT 16.5*). In addition, some proteins that are degraded when expressed in the soluble fraction are quite stable as inclusion bodies. Once purified, protein in inclusion bodies can be solubilized by denaturation with guanidine·HCl or urea, and then can often be refolded by dialyzing away the denaturant. A problem, however, with denaturation/renaturation is that the yield of properly refolded protein is variable and sometimes quite low; some proteins, especially large ones, cannot be properly refolded at all (see *UNIT 16.5*).

If expression of a particular fusion protein produces insoluble aggregates and a soluble protein is required, there are several things to try. One important variable is temperature; for reasons not well understood, higher temperatures (37° and 42°C) promote inclusion-body formation and lower temperatures (30°C) inhibit it (Bishai et al., 1987; Schein, 1989). Another variable is the level of expression; sometimes lowering the expression level can increase the proportion of protein that is soluble. A third variable is the strain background of the cells bearing the expression vector; large differences in the proportion of a particular expressed protein that is soluble are seen among different strains (M. Southworth, S. Levitt, and F. Perler, unpub. observ.; it is not known which of the genetic differences between the strains is responsible for the differences in solubility). Finally, it is worth noting that changes in the carrier protein can affect the solubility of an expressed fusion protein (La Vallie et al., 1993).

STABILITY OF THE EXPRESSED PROTEIN

Stability problems are often encountered when foreign proteins, especially eukaryotic proteins, are expressed in *E. coli*. The carrier protein can sometimes stabilize an expression fusion protein (Lee et al., 1984). Sometimes, however, the expressed protein is degraded but the carrier protein is not. Moreover, fusion proteins are sometimes cleaved in vivo at the fusion joint between the carrier and expressed portions of the fusion, which obviously creates problems if the carrier protein is to be used as an aid in purification. These facts about fusion proteins are consistent with a model in which the carrier and the rest of the protein form independent domains. In this view, it can be imagined that there are cases where the carrier domain folds correctly and the expressed protein does not (and is degraded). There are also cases where both domains fold correctly but the joint region between them is sensitive to one or more *E. coli* proteases.

Approaches that have been used to stabilize fusion proteins are generally the same as those used to stabilize nonfusion proteins. One method is to arrange for the fusion protein to be expressed as insoluble aggregates. Another method is to use *E. coli* strains deficient in known proteases. For example, a *lon htpR* double-mutant strain—which is deficient in several cytoplasmic proteases—shows reduced degradation of unstable proteins (Baker et al., 1984; I. Hall and P. Riggs, unpub. observ.). Similarly, the *degP* mutant has been shown to stabilize fusion proteins in the periplasm (Strauch and Beckwith, 1988) and *ompT* mutants have proven useful in preventing cleavage between exposed basic residues (e.g., Arg-Arg) in several nonfusion proteins during preparation of crude extracts (Grodberg and Dunn, 1988; Sugimura and Higashi, 1988). Finally, the stability of a particular fusion can vary even among different "wild-type" lab strains, perhaps due to uncharacterized differences in protease levels among the strains (I. Hall, P. Riggs, M. Southworth, S. Levitt, and F. Perler, unpub. observ.).

CLEAVAGE OF FUSION PROTEINS TO REMOVE THE CARRIER

The use of fusion proteins is growing rapidly for the many reasons described above. The various systems described in the following units have been used to produce many different kinds of proteins ranging from enzymes and growth factors to transmembrane receptors and DNA binding proteins. Often it is advantageous to remove the carrier protein moiety from the protein of interest to facilitate biochemical and functional analyses. Several methods for site-specific cleavage of fusion proteins have been developed (*UNIT 16.4B*). The choice of method is usually determined by the composition, sequence, and physical characteristics of the particular protein. Chemical cleavage of fusion proteins can be accomplished with reagents such as cyanogen bromide (Met↓, Itakura et al., 1977), 2-(2-nitrophenylsulphenyl)-3-methyl-3′-bromoindolenine (BNPS-skatole, Trp↓, Dykes et al., 1988), hydroxylamine (Asn↓Gly, Bornstein and Balian, 1977), or low pH (Asp↓Pro, Szoka et al., 1986). Chemical cleavage procedures tend to be inexpensive and efficient, and often can be accomplished under denaturing conditions to cleave otherwise insoluble fusion proteins (Szoka et al., 1986). However, their use is hampered by the likely occurrence of cleavage sites in the protein of interest, along with the propensity for side reactions that result in unwanted modifications to the protein. As an alternative to chemical methods, enzymatic cleavage procedures are desirable for their relatively mild reaction conditions and, most importantly, for the high degree of specificity exhibited by some proteases commonly used for this purpose. Among the useful enzymes are factor Xa (Nagai and

Thøgersen, 1984, 1987; Gardella et al., 1990), thrombin (Smith and Johnson, 1988; Gearing et al., 1989), enterokinase (Dykes et al., 1988; LaVallie et al., 1993), renin (Haffey at al., 1987), and collagenase (Germino and Bastia, 1984). All of these enzymes have extended substrate recognition sequences (up to 7 amino acids in the case of renin), which greatly reduces the likelihood of unwanted cleavages elsewhere in the protein. Of the above-mentioned proteases, factor Xa and enterokinase are most useful in this application because they cleave on the carboxy-terminal side of their respective recognition sequences, allowing the release of fusion partners containing their authentic amino-termini.

UNITS 16.5, 16.6, 16.7 & 16.8 describe five different fusion protein vector systems; of these, only three include recognition sites for interdomain cleavage. The MBP fusion system (*UNIT 16.6*) provides a factor Xa cleavage site. The GST fusion system (*UNIT 16.7*) includes vectors that contain either a thrombin cleavage site, a factor Xa cleavage site, or an Asp-Pro acid cleavage site. The Trx fusion system (*UNIT 16.8*) uses an enterokinase cleavage site. *UNIT 16.4B* describes fusion protein cleavages in detail, including specific protocols for cleaving fusion proteins produced with each of the aforementioned vector systems, along with methodologies for the site-specific cleavage of proteins using various chemical reagents.

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Enzymatic and Chemical Cleavage of Fusion Proteins

The use of gene fusion expression systems has become an increasingly popular method of producing foreign proteins in *Escherichia coli*. This popularity is due in large part to the development of fusion systems that are capable of producing large amounts of fusion protein in a soluble form. The maltose-binding protein (MBP, *UNIT 16.6*), glutathione-S-transferase (GST, *UNIT 16.7*), and thioredoxin (Trx, *UNIT 16.8*) fusion systems have proven singularly successful in producing properly folded and biologically active proteins. Each of these systems also provides convenient methods for specific purification of the fusion protein from cellular contaminants. As a result, proteins produced using these systems are readily amenable to the study of their biological activities and/or interactions. As a consequence of the popularity of fusion protein expression strategies, the ability to cleave the N-terminal fusion "carrier" protein from the C-terminal protein of interest has become increasingly important.

This unit provides protocols for some commonly used methods of site-specific cleavage of fusion proteins. The first three protocols describe enzymatic cleavage of proteins using proteases that display highly restricted specificities, which greatly decrease the likelihood that unwanted secondary cuts will occur. The first basic protocol describes the use of factor Xa, a mammalian serine protease that cleaves following the sequence Ile-Glu(or Asp)-Gly-Arg↓. This protocol can be applied to fusion proteins produced with either the MBP (*UNIT 16.6*) or the GST system (pGEX3X vector; *UNIT 16.7*); both systems utilize expression vectors that encode a factor Xa cleavage site. A support protocol describes conditions for denaturing proteins for factor Xa cleavage if necessary. The next two protocols (first and second alternate protocols) describe cleavage with thrombin, a site-specific protease that recognizes the sequence Leu-Val-Pro-Arg↓Gly-Ser in one of the GST expression vectors (pGEX2T) described in *UNIT 16.7*. The third enzymatic cleavage protocol (third alternate protocol) uses enterokinase (enteropeptidase), a mammalian intestinal protease that cleaves following the sequence Asp-Asp-Asp-Asp-Lys↓. The Trx fusion vectors pTRXFUS and hpTRXFUS (*UNIT 16.8*) encode an enterokinase cleavage site immediately prior to their fusion junctions.

Three additional protocols describe cleavage of fusion proteins with chemical reagents as an alternative to enzymatic cleavage. These have some advantages. Though these methods may require modification of the fusion protein so a scissile or labile bond resides at the desired point of cleavage, they may be useful for cleaving fusion proteins with solubility problems or those that are otherwise refractory to enzymatic cleavage. Chemical cleavage methods have the disadvantage of being less specific, and it is necessary to ensure that a susceptible peptide bond does not exist in the protein of interest. The first of the chemical cleavage protocols (second basic protocol) uses cyanogen bromide to cleave after methionine residues. The second method (fourth alternate protocol) uses hydroxylamine to specifically cleave between asparagine and glycine residues. The final chemical cleavage protocol (fifth alternate protocol) cleaves fusion proteins by exploiting the lability of the Asp-Pro bond at low pH.

BASIC PROTOCOL 1

ENZYMATIC CLEAVAGE OF FUSION PROTEINS WITH FACTOR Xa

Fusion proteins that have been produced with the MBP fusion vectors pMAL-c2, pMAL-p2, or the GST fusion vector pGEX3X contain a recognition sequence for coagulation factor Xa encoded in the DNA immediately preceding the polylinker cloning site. Fusion proteins produced in other systems must be adapted to encode this recognition sequence. It is important to note that factor Xa will not cleave if a proline residue follows the arginine of the recognition sequence. Purification of the fusion protein prior to cleavage is recommended to minimize degradation of the product by nonspecific cellular proteases during incubation with factor Xa protease. Prior purification of the fusion protein also allows subsequent isolation of the cleaved product by simply repeating whatever affinity purification step was performed to purify the fusion protein. This step now removes the fusion partner.

Factor Xa is typically added to the fusion protein substrate at a ratio of 1% to 2% (w/w). However, cleavage efficiency varies depending upon the individual fusion, and ratios ranging from 0.1% to 5% may be effective. Incubation times can be from 1 hr to several days at either room temperature or 4°C. A support protocol describes denaturing and renaturing soluble fusion proteins that do not cleave well under the standard native cleavage conditions. This procedure should be considered a last resort because of the uncertainty of regaining properly folded protein and the inevitable decrease in overall yield.

Materials

*For recipes, see **Reagents and Solutions** in this unit (or cross-referenced unit); for common stock solutions, see **APPENDIX 2**; for suppliers, see **APPENDIX 4**.*

1 mg/ml fusion protein
200 µg/ml factor Xa (New England Biolabs) in reaction buffer (see step 1)
2× SDS sample buffer (*UNIT 10.2*)
Boiling water bath

Additional reagents and equipment for SDS-PAGE (*UNIT 10.2*)

1. Prepare two small-scale trial reactions to determine optimum incubation time as follows:

   Reaction 1: 20 µl of 1 mg/ml fusion protein with 1 µl of 200 µg/ml factor Xa.
   Reaction 2: 5 µl of 1 mg/ml fusion protein and no factor Xa (mock digestion).

   Incubate at room temperature.

   Fusion protein in column buffer from amylose-resin purification (UNIT 16.6; with 1 mM $CaCl_2$) or in PBS from glutathione-agarose purification (UNIT 16.7) is suitable for factor Xa digestion; otherwise the protein should be prepared in 20 mM Tris·Cl (pH 8.0)/1 mM $CaCl_2$/100 mM NaCl.

   Although most fusion proteins could be kept at 4°C, any remaining fusion protein solution can be stored at –70°C, in 10% glycerol, until used in step 6.

2. At 2, 4, 8, and 24 hr, remove 5-µl aliquots of the factor Xa reaction, add 5 µl of 2× SDS sample buffer, and freeze at –20°C.

3. At 24 hr mix 5 µl mock digestion with 5 µl of 2× SDS sample buffer.

4. Mix 5 µl of original fusion protein solution with 5 µl of 2× SDS sample buffer (uncut control).

5. Heat all samples 10 min in a boiling water bath and load onto an SDS-polyacrylamide gel. Evaluate extent of cleavage to determine correct incubation time.

   Gel composition and running conditions will be determined by the size of the fusion protein.

   If only partial cleavage is evident, increase amount of enzyme and/or incubation time. If no cleavage is apparent, proceed to the next support protocol.

6. Once satisfactory cleavage conditions have been determined, scale up the trial reaction for the remainder of the fusion protein sample, saving a small amount of uncleaved fusion protein for comparison purposes. Monitor the extent of cleavage by SDS-PAGE.

   The cleavage products can be separated using any of the support protocols in UNIT 16.6.

DENATURING A FUSION PROTEIN FOR FACTOR Xa CLEAVAGE

SUPPORT PROTOCOL

It has been observed that some fusion proteins are resistant to cleavage with factor Xa. This problem can sometimes be alleviated by denaturing the fusion protein, renaturing it, and then incubating it with protease. The following protocol has been adapted from New England Biolabs' recommendations for MBP fusion proteins, and should be applicable to any fusion protein that contains an inaccessible factor Xa cleavage site. Denaturation is accomplished by incubating the fusion protein in 6 M guanidine·HCl followed by dialysis against the reaction buffer.

Additional Materials

*For recipes, see **Reagents and Solutions** in this unit (or cross-referenced unit); for common stock solutions, see **APPENDIX 2**; for suppliers, see **APPENDIX 4**.*

20 mM Tris·Cl (pH 7.4)/6 M guanidine·HCl
20 mM Tris·Cl (pH 8.0)/1 mM $CaCl_2$

1. Dialyze fusion protein for ≤4 hr against ≥10 vol of 20 mM Tris·Cl (pH 7.4)/6 M guanidine·HCl, or add guanidine·HCl to the fusion protein to give a final concentration of 6 M.
2. Dialyze the sample for 4 hr against 100 vol of 20 mM Tris·Cl (pH 8.0)/1 mM $CaCl_2$.
3. Repeat the second dialysis for an additional 4 hr against 100 vol fresh buffer.

   This denaturation procedure is intended to allow better accessibility of the cleavage site to factor Xa before the protein can completely reassume its former protease-resistant conformation. Therefore, it is best to proceed with the cleavage reaction immediately following dialysis. However, rapid removal of denaturant sometimes results in precipitation of the protein; in these cases, gradual removal of denaturant by stepwise dialysis against 2-fold dilutions of the guanidine·HCl solution may keep the protein from precipitating. Alternatively, the fusion protein remaining in solution after rapid dialysis precipitation can be recovered and cleaved, and the insoluble material discarded.

4. Proceed with step 1 of the first basic protocol for factor Xa cleavage.

ALTERNATE PROTOCOL 1

ENZYMATIC CLEAVAGE OF FUSION PROTEINS WITH THROMBIN

Thrombin is a mammalian serine protease that cleaves in a trypsin-like manner; that is, it cleaves after arginine and lysine residues. However, thrombin displays distinct subsite preferences, with optimum cleavage occurring at sites containing P4-P3-Pro-Arg↓P1′-P2′ (where P4 and P3 are hydrophobic amino acids and P1′ and P2′ are nonacidic amino acids). The GST fusion system (*UNIT 16.7*) utilizes a vector that encodes a cleavage site with this restricted specificity (Leu-Val-Pro-Arg↓Gly-Ser). GST fusion proteins expressed with the pGEX2T vector (Fig. 16.7.1) can be cleaved with thrombin either after affinity purification on glutathione-agarose, or alternatively, while still bound to the affinity matrix. The following alternate protocols describe both methods for cleaving the fusion protein: first, cleavage of fusion proteins with thrombin in solution, an approach that is applicable to any fusion protein containing a thrombin recognition sequence; and second, thrombin cleavage of GST fusion proteins bound to glutathione-agarose. The latter technique is preferred for GST fusion proteins because is it faster and usually requires less work. However, the approach may not always work: some proteins may become insoluble when separated from the GST carrier, thus complicating their physical separation from the affinity matrix. In this case, the first alternate protocol should be used.

Additional Materials

*For recipes, see **Reagents and Solutions** in this unit (or cross-referenced unit); for common stock solutions, see **APPENDIX 2**; for suppliers, see **APPENDIX 4**.*

Thrombin cleavage buffer (see recipe)
Heparin, sodium salt (with ≥140 U/mg activity, Sigma; optional)
Thrombin (human, with ~3000 U/mg activity; Sigma or Boehringer Mannheim)

1. Prepare two pilot cleavage reactions to determine optimal reaction conditions as follows:

   Reaction 1: 20 µl of 1 mg/ml fusion protein solution (in appropriate buffer) and 0.2 µg thrombin.
   Reaction 2: 5 µl of 1 mg/ml fusion protein solution only (mock digestion).

   Incubate at 25°C.

   GST fusion protein that has been eluted from glutathione-agarose in 50 mM Tris·Cl (pH 7.5)/5 mM reduced glutathione can be used after addition of NaCl to 150 mM and $CaCl_2$ to 2.5 mM and adjustment of the protein concentration to 1 mg/ml. Other fusion proteins can be resuspended or dialyzed in thrombin cleavage buffer (without glutathione) for subsequent cleavage.

   Addition of 10 µM heparin to the cleavage reaction is optional. It has been reported (Chang, 1985) that this increases the rate of some cleavages by 10% to 50%, apparently due to a direct interaction with the enzyme.

2. At 30 min, 1, 2, and 4 hr, remove 5 µl from the thrombin reaction and mix with 5 µl of 2× SDS sample buffer. Freeze at –20°C.
3. At the 4 hr time point, add 5 µl of 2× SDS sample buffer to the mock digestion.
4. Mix 5 µl of original fusion protein solution with 5 µl of 2× SDS sample buffer (untreated control).
5. Boil all samples 10 min and load on an SDS-polyacrylamide gel to analyze sample stability and efficiency of cleavage.
6. Use those conditions determined empirically to be best for cleaving the fusion protein to scale up the cleavage reaction for the desired quantity of protein.