12/02/97

Class	Subclass
435	6

ISSUE CLASSIFICATION

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[redacted]

6051379

UTILITY SERIAL NUMBER	PATENT DATE APR 18 2000	PATENT NUMBER 6051379

SERIAL NUMBER	FILING DATE	CLASS	SUBCLASS	GROUP ART UNIT	EXAMINER
08/984,034	12/02/97	435	6	~~1643~~ 1634 1655	~~Zitomer~~ Arthur

APPLICANTS: JENNIFER LEE LESCALLETT, GREAT FALLS, VA; TAMMY LAWRENCE, LAUREL, MD; ANTONETTE PREISINGER ALLEN, SEVERN, MD; SHERI JON OLSON, FALLS CHURCH, VA; DENIS BERNADETTE THURBER, SILVER SPRING, MD; MARGA BELLE WHITE, FREDERICK, MD.

CONTINUING DATA********************
VERIFIED

PROVISIONAL APPLICATION 60/059,595
FILED 9/23/97

LBA

FOREIGN APPLICATIONS**********
VERIFIED

none
LBA

CPA

FOREIGN FILING LICENSE GRANTED 03/03/98 ***** SMALL ENTITY *****

Foreign priority claimed / 35 USC 119 conditions met / Verified and Acknowledged	AS FILED	STATE OR COUNTRY	SHEETS DRWGS.	TOTAL CLAIMS	INDEP. CLAIMS	FILING FEE RECEIVED	ATTORNEY'S DOCKET NO.
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ADDRESS: BAKER & BOTTS
30 ROCKEFELLER PLAZA
NEW YORK NY 10112

TITLE: CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2

U.S. DEPT. OF COMM./ PAT. & TM—PTO-436L (Rev.12-94)

PARTS OF APPLICATION FILED SEPARATELY

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CLAIMS ALLOWED

Total Claims	Print Claim
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LISA B. ARTHUR
PRIMARY EXAMINER
GROUP ~~1800~~ 1600

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DRAWING

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PREPARED FOR ISSUE

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(Rev. 8/92)

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ISSUE FEE IN FILE

(FACE)

Briefed in 600

PATENT APPLICATION

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APPROVED FOR LICENSE

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Date Entered or Counted			Date Received or Mailed	
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/	4.	Interview summary	6/10/98	
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APPLICATION SERIAL NUMBER
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ORIGINAL CLASSIFICATION

CLASS	SUBCLASS
435	6

CROSS REFERENCE(S)

CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)			
536	24.31	24.33		

INTERNATIONAL CLASSIFICATION

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C	0	7	H	21 / 04
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ASSISTANT EXAMINER (PLEASE STAMP OR PRINT FULL NAME)

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Lisa Arthur

PTO 270
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ISSUE CLASSIFICATION SLIP

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

Final	Original	120 / 98	110 / 99	120 / 99
	1	✓	✓	
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Final	Original	120 / 98	110 / 99	120 / 99
26	51		✓	=
27	52			
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SYMBOLS

✓	Rejected
=	Allowed
- (Through numberal)	Canceled
+	Restricted
N	Non-elected
I	Interference
A	Appeal
O	Objected

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SEARC D

Class	Sub.	Date	Exmr.
536	24.33	7/9/99	LBA
"	24.31	↓	↓
435	6		
435	91.2		
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435	320.1	↓	↓
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Reviewed provisional 60/059,595		
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BRCA2 and cancer	↓	↓
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updated	3/16/99	LBA
updated sequen search on SEQ ID NOs 1-24	12/13/99	LBA
	↓	↓

INTERFERENCE SEARCHED

Class	Sub.	Date	Exmr.
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530	24.3		
536	31		
435	12	↓	↓

PATENT APPLICATION SERIAL NO. _____

U.S. DEPARTMENT OF COMMERCE
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02/03/1998 KHARLING 00000049 08984034
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03 FC:203 605.00 OP

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BAKER & BOTTS, L.L.P.

BAKER & BOTTS P.
30 ROCKEFELLER PLAZA
NEW YORK, NEW YORK 10112-0228
212 705-5000
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PATENT

UTILITY PATENT APPLICATION TRANSMITTAL
(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No. A31420 - 2880/00002

First Named Inventor Jennifer L. Lescallet

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Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

Sir:

Enclosed herewith for filing is a patent application of JENNIFER L. LESCALLET, ANTONETTE C.P. ALLEN, TAMMY LAWRENCE, SHERI J. OLSON, DENISE B. THURBER, and MARGA B. WHITE entitled CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2

which includes:

[X] Specification 88 Total Pages
[] Drawing(s) ____ Total Sheets
[X] Combined Declaration and Power of Attorney 4 Total Pages
  [X] Newly executed (original or copy)
  [] Copy from a prior application
  (for continuation/divisional only)

[] Continuation [] Divisional [] Continuation-In-Part (CIP)
of prior application No. ____/_____

[X] An Assignment of the invention to
  [] is attached. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
  [X] will follow.

[] Certified Copy of Priority Document(s) Country_____, No. _____, filed _____.

[X] Small Entity Statement(s)
  [] Small Entity Statement filed in prior application. Status still proper and desired.

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PATENT

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[] Information Disclosure Statement (IDS) PTO-1449
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Basic Fee					$395			$790
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Ind. Claims	8	-3 =	5	x $41 =	$205		x $82 =	$400
Multiple Dependent Claim				+ $135 =	$		+$270=	$
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[X] Basic filing fee $1249

[] Recording Assignment
[$40.00; 37 CFR 1.21(h)] $

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[X] The Commissioner is hereby authorized to charge payment of any additional filing fees required under 37 CFR 1.16, 1.17, and 1.21(h) associated with this communication or credit any overpayment to Deposit Account No. 02-4377. Two copies of this sheet are enclosed.

BAKER & BOTTS, L.L.P.

By Rochelle K. Seide

Rochelle K. Seide

PTO Registration No. 32,300

Enclosures

ABSTRACT OF THE DISCLOSURE

New mutations have been found in the BRCA2 gene. The mutations are located at nucleotide numbers 2192, 3772, 5193, 5374, 6495 or 6909 of the published nucleotide sequence of BRCA2 gene. A process for identifying a sequence variation in a BRCA2 polynucleotide sequence is disclosed. The identification process includes allele specific sequence-based assays of known sequence variations. The methods can be used for efficient, and accurate detection of a mutation in a test BRCA2 gene sample.

Attorney Docket No: PA-0107

CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2

This application is in part based on provisional patent application 60/059,595 filed September 23, 1997, the contents are incorporated by reference.

FIELD OF THE INVENTION

This invention relates to the breast cancer succeptibility gene BRCA2. More specifically, this invention detects germline mutations of the BRCA2 gene that are associated with a predisposition to breast, ovarian and asociated cancers. Methods and reagents for detecting the presence of these mutations are included.

BACKGROUND OF THE INVENTION

BRCA2, located on chromosome 13q12-q13, consists of over 70kb of genomic DNA. The coding sequence produces a protein of 3,418 amino acids. Although most of the exons are small, exons 10 and 11 represent approximately 60% of the entire coding region. BRCA2 is thought to be a tumor suppressor gene asociated with breast and ovarian cancer. Thus mutantions which form an altered tumor suppressor or altered concentrations of tumor suppressor may be indicative of a higher succeptibility to certain cancers.

The nucleotide sequence for at least one BRCA2 gene is known and is reported in GENBANK accession Number U43746. The BRCA2 gene sequence is available on the Breast Cancer Information Core.

Germline mutations of BRCA2 are predicted to account for approximately 35% of families with multiple case, early onset female breast cancer, and they are also associated with an increased risk of male breast cancer, ovarian cancer, prostrate cancer and pancreatic cancer.

The location of one or more mutations of the BRCA2 gene provides a promising approach to reducing the high incidence and mortality associated with breast and ovarian cancer through the early detection of women at high risk. These women, once identified, can be targeted for more aggressive prevention programs. Screening is carried out by a variety of methods which include karyotyping, probe binding and DNA sequencing. In such cases where one or only a few known mutations are responsible for the disease, such as testing family members, methods for detecting the mutations are targeted to the site within the gene at which they are known to occur.

Many mutations and normal polymorphisms have already been reported in the BRCA2 gene. A world wide web site has been built to facilitate the detection and characterization of alterations in breast cancer susceptibility genes. Such mutations in BRCA2 can be accessed through the Breast Cancer Information Core at:
HTTP://www.nchgr.nih.gov/dir/lab_transfer/bic.

While mutations occur throughtout the BRCA2 gene, there is a need for a high sample number (throughput), sensitivity, accuracy and cost effectiveness. Identification of mutations of the BRCA2 gene would allow more widespread diagnostic screening for hereditary breast and ovarian cancer than is currently possible and permit identification of functional areas deduced from the mutational spectrum observed.

SUMMARY OF THE INVENTION

The present invention is based on the discovery of six mutations in the BRCA2 gene sequence which is associated with susceptibility to and development of breast and ovarian cancer. Specifically, mutations located at nucleotide numbers 2192, 3772, 5193, 5374, 6495 and 6909 have been discovered.

It is an object of the invention to provide a method for determining a predisposition or higher susceptibility to breast, ovarian and other cancers.

It is another object of the invention to provide primers for detecting and amplifying a region of DNA which contains the BRAC2 mutations.

It is another object of the invention to provide probes for detecting a region of DNA which contains the BRAC2 mutations.

It is a further object of the invention to provide a method of characterizing and classifying a tumor and determining a therapy dependant upon the type of mutation(s) present.

It is also an object of the present invention to provide a mutant BRCA2 gene and expressed mutant protein for drug development, gene therapy and other uses to prevent or amelorate the effects of or resulting from the mutant BRCA2 gene.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

For defining the present invention, the following nomenclature is used to describe the mutation due to an inconsistency in the published literature. Beaudet et al, Human Mutations, 2: 245-248 (1993), Antonarakis et al, Human Mutations, 4: 166 (1994), Cotton, Human Mutations, 8: 197-202

(1996), and Beutler et al, Human Mutations, 8: 203-206 (1996). In defining the mutation, the number indicates the nucleotide number corresponding to the BRCA2 gene sequence where the mutation first occurs. Other BRCA2 sequences (haplotypes) which are polymorphisms or genetic variations of BRCA2 may used, in which a corresponding mutation at the corresponding nucleotide number are present. Different sequence variations in a normal BRCA1 gene have been discovered previously by the inventors (U.S. Patent 5,654,155) and sequence variations in a normal BRCA2 gene sequence are expected. Also note Shattuck-Eidens, et al, Journal of the American Medical Association, 278: p. 1242 (1997). Generally, the sense strand is referred to. For simplified identification purposes of this application, reference is to the BRCA2 sequence referenced above, however the invention is equally applicable to all of the normal BRCA2 sequences.

Insertion mutations are indicated by "ins" and deletion mutations are indicated by "del". The letters after "ins" or "del" refer to the nucleotide(s) which were inserted or deleted. Insertions and deletions above two nucleotides are indicated by the number of nucleotides inserted or deleted. When the mutation results in one nucleotide being substituted for another, the nucleotide of the BRCA2 gene sequence is placed to the left of the number and the nucleotide found in the mutation is placed to the right of the number.

The first mutation is referred to as C2192G. This mutation or genetic alteration causes a change in nucleotide number 2192 from C to G resulting in codon 655 being changed from proline to arginine. Any amino acid change can have a dramatic change in biological activity. Some people believe that since proline can form a turn in the chain of amino acids

in the protein, the removal of this turn, particularly when substituted with a charged amino acid may change the three dimentional configuration of the protein or at least may negatively affect on the biological activity of the resulting protein.

The second mutation is referred to as 3772delTT. This mutation deletes TT at nucleotide number 3772 causing a frameshift mutation and forming an in-frame stop codon at codon 1182. It has been demonstrated that a truncated, and most likely non-functional, protein has been produced by this mutation.

The third mutation is referred to as C5193G. This mutation substitutes G for C at nucleotide number 5193 causing a stop codon (TAG) to be formed at codon 1655. It has been demonstrated that a truncated, and most likely non-functional, protein has been produced by this mutation.

The fourth mutation is referred to as 5374del4. This mutation deletes TATG at nucleotide number 5374 causing a frameshift mutation and forming an in-frame stop at codon 1723. It has been demonstrated that a truncated, and most likely non-functional, protein has been produced by this mutation.

The fifth mutation is referred to as 6495delGC. This mutation deletes GC at nucleotide number 6495 causing a frameshift mutation and forming an in-frame stop codon at codon 2090. It has been demonstrated that a truncated, and most likely non-functional, protein has been produced by this mutation.

The sixth mutation is referred to as 6909insG. This mutation inserts a G at nucleotide number 6909 causing a frameshift mutation and forming an in-frame stop codon at

codon 2232. It has been demonstrated that a truncated, and most likely non-functional, protein has been produced by this mutation.

The presence of truncated proteins was demonstrated by expression of overlapping portions of the mutant genes and measuring molecular weight by gel electrophoresis.

Useful DNA molecules according to the present invention are those which will specifically hybridize to BRCA2 sequences in the region of the C2192G, 3772delTT, C5193G, 5374del4, 6495delGC or 6909insG mutations. Typically these DNA molecules are 17 to 20 nucleotides in length (longer for large insertions) and have the nucleotide sequence corresponding to the region of the mutations at their respective nucleotide locations on the BRCA2 gene sequence. Such molecules can be labeled, according to any technique known in the art, such as with radiolabels, fluorescent labels, enzymatic labels, sequence tags, biotin, other ligands, etc.

According to another aspect of the invention, the DNA molecules, or oligonucleotides, contain one or more of the specific mutations. Generally it is preferred for each DNA probe to encompass only one mutation. Such molecules may be labeled and can be used as allele-specific oligonucleotide probes to detect the mutation of interest.

Polynucleotide containing biological samples, such as blood, can be tested to determine whether the BRCA2 gene contains one of the specific mutations listed above. To amplify the BRCA2 gene, one may use polymerase chain reaction (PCR) using primers which hybridize to the ends of the exons or to the introns flanking the exons. In the situation of exon 11, the exon is so large that using plural pairs of

primers to amplify overlapping regions is preferred. Such was actually used in the Examples below.

Amplification may also be performed by a number of other techniques such as by cloning the gene and linking the BRCA2 gene or fragments thereof in the sample to a vector. "Shot gun" cloning is particularly preferred. For the purposes of this application, a vector may be any polynucleotide containing system which induces replication such as a plasmid, cosmid, virus, transposon, or portions thereof.

In one embodiment of the invention a pair of isolated oligonucleotide primers are provided.

BRCA2-11F 5'TGG TAC TTT AAT TTT GTC ACT T3' SEQ ID NO:1
BRCA2-11R 5'TGC AGG CAT GAC AGA GAA T3' SEQ ID NO:2

The designation BRCA2-11 refers to a sequence in or near exon 11 of the BRCA2 gene. F and R refer to forward and reverse.

The oligonucleotide primers are useful in directing amplification of a target polynucleotide prior to sequencing. These unique BRCA2 exon 11 oligonucleotide primers were used to scan the BRCA2 gene to find the mutations. From the sequence information, the probes were designed and produced to assay for the mutation based upon identification of the C2192G mutation.

In another embodiment of the invention a pair of isolated allele specific oligonucleotide probes are provided.

5'TGA AGA ACC AAC TTT GT3' SEQ ID NO:3
5'TGA AGA ACG AAC TTT GT3' SEQ ID NO:4

These allele specific oligonucleotides are useful in diagnosis of a subject at risk of having breast or ovarian cancer. The allele specific oligonucleotides hybridize with a target polynucleotide sequence containing the C2192G mutation. 5'TGA AGA ACC AAC TTT GT3', SEQ ID NO:3, hybridizes preferentially to the wildtype sequence and is useful as a control sequence. 5'TGA AGA ACG AAC TTT GT3', SEQ ID NO:4, is designed to hybridize preferentially to the mutant sequence.

In a second embodiment of the invention a pair of isolated oligonucleotide primers are provided.

BRCA2-11F 5'CTC AGA TGT TAT TTT CCA AGC3'	SEQ ID NO:5
BRCA2-11R 5'CTG TTA AAT AAC CAG AAG CAC3'	SEQ ID NO:6

The oligonucleotide primers are useful in directing amplification of a target polynucleotide prior to sequencing. These unique BRCA2 exon 11 oligonucleotide primers were used to scan the BRCA2 gene to find the mutations. From the sequence information, the probes were designed and produced to assay for the mutation based upon identification of the the 3772delTT mutation.

In another embodiment of the invention a pair of isolated allele specific oligonucleotides are provided.

5'GCA AGC AAT TTG AAG GT3'	SEQ ID NO:7
5'GCA AGC AAT GAA GGT AC3'	SEQ ID NO:8

These allele specific oligonucleotides are useful in diagnosis of a subject at risk of having breast or ovarian cancer. The allele specific oligonucleotides hybridize with a target polynucleotide sequence containing the 3772delTT

mutation. 5'GCA AGC AAT TTG AAG GT3', SEQ ID NO:7, hybridizes preferentially to the wildtype sequence and is useful as a control sequence. 5'GCA AGC AAT GAA GGT AC3', SEQ ID NO:8, is designed to hybridize preferentially to the mutant sequence.

In a third embodiment of the invention a pair of isolated oligonucleotide primers are provided.

BRCA2-11F 5'GCA AAG ACC CTA AAG TAC AG3', SEQ ID NO:9
BRCA2-11R 5'CAT CAA ATA TTC CTT CTC TAA G3', SEQ ID NO:10

The oligonucleotide primers are useful in directing amplification of a target polynucleotide prior to sequencing. These unique BRCA2 exon 11 oligonucleotide primers were used to scan the BRCA2 gene to find the mutations. From the sequence information, the probes were designed and produced to assay for the mutation based upon identification of the C5193G mutation.

In another embodiment of the invention a pair of isolated allele specific oligonucleotides are provided.

5'ACT TGT TAC ACA AAT CA3', SEQ ID NO:11
5'ACT TGT TAG ACA AAT CA3', SEQ ID NO:12

These allele specific oligonucleotides are useful in diagnosis of a subject at risk of having breast or ovarian cancer. The allele specific oligonucleotides hybridize with a target polynucleotide sequence containing the C5193G mutation. 5'ACT TGT TAC ACA AAT CA3', SEQ ID NO:11, hybridizes preferentially to the wildtype sequence and is useful as a control sequence. 5'ACT TGT TAG ACA AAT CA3', SEQ ID NO:12,

is designed to hybridize preferentially to the mutant sequence.

In a fourth embodiment of the invention a pair of isolated oligonucleotide primers are provided.

BRCA2-11F 5'GAA AAT TCA GCC TTA GC3' SEQ ID NO:13
BRCA2-11R 5'ATC AGA ATG GTA GGA AT3' SEQ ID NO:14

The oligonucleotide primers are useful in directing amplification of a target polynucleotide prior to sequencing. These unique BRCA2 exon 11 oligonucleotide primers were used to scan the BRCA2 gene to find the mutations. From the sequence information, the probes were designed and produced to assay for the mutation based upon identification of the 5374del4 mutation.

In another embodiment of the invention a pair of isolated allele specific oligonucleotides are provided.

5'ATT ATT TGT ATG AAA AT3' SEQ ID NO:15
5'ATT ATT TGA AAA TAA TT3' SEQ ID NO:16

These allele specific oligonucleotides are useful in diagnosis of a subject at risk of having breast or ovarian cancer. The allele specific oligonucleotides hybridize with a target polynucleotide sequence containing the 5374del4 mutation. 5'ATT ATT TGT ATG AAA AT3', SEQ ID NO:15, hybridizes preferentially to the wildtype sequence and is useful as a control sequence. 5'ATT ATT TGA AAA TAA TT3', SEQ ID NO:16, is designed to hybridize preferentially to the mutant sequence.

In a fifth embodiment of the invention a pair of isolated oligonucleotide primers are provided.

BRCA2-11F 5'TAC AGC AAG TGG AAA GC3' SEQ ID NO:17
BRCA2-11R 5'AAG TTT CAG TTT TAC CAA T3' SEQ ID NO:18

The oligonucleotide primers are useful in directing amplification of a target polynucleotide prior to sequencing. These unique BRCA2 exon 11 oligonucleotide primers were used to scan the BRCA2 gene to find the mutations. From the sequence information, the probes were designed and produced to assay for the mutation based upon identification of the 6495delGC mutation.

In another embodiment of the invention a pair of isolated allele specific oligonucleotides are provided.

5'GAA CTG AGC ATA GTC TT3' SEQ ID NO:19
5'GAA CTG AAT AGT CTT CA3' SEQ ID NO:20

These allele specific oligonucleotides are useful in diagnosis of a subject at risk of having breast or ovarian cancer. The allele specific oligonucleotides hybridize with a target polynucleotide sequence containing the 6495delGC mutation. 5'GAA CTG AGC ATA GTC TT3', SEQ ID NO:19, hybridizes preferentially to the wildtype sequence and is useful as a control sequence. 5'GAA CTG AAT AGT CTT CA3', SEQ ID NO:20, is designed to hybridize preferentially to the mutant sequence.

In a sixth embodiment of the invention a pair of isolated oligonucleotide primers are provided.

BRCA2-11F 5'ACT TTT TCT GAT GTT CCT GTG3' SEQ ID NO:21

BRCA2-11R 5'TAA AAA TAG TGA TTG GCA ACA3' SEQ ID NO:22

The oligonucleotide primers are useful in directing amplification of a target polynucleotide prior to sequencing. These unique BRCA2 exon 11 oligonucleotide primers were used to scan the BRCA2 gene to find the mutations. From the sequence information, the probes were designed and produced to assay for the mutation based upon identification of the 6909insG mutation.

In another embodiment of the invention a pair of isolated allele specific oligonucleotides are provided.

5'CAG AAG CAG TAG AAA TT3' SEQ ID NO:23

5'CAG AAG CAG GTA GAA AT3' SEQ ID NO:24

These allele specific oligonucleotides are useful in diagnosis of a subject at risk of having breast or ovarian cancer. The allele specific oligonucleotides hybridize with a target polynucleotide sequence containing the 6909insG mutation. 5'CAG AAG CAG TAG AAA TT3', SEQ ID NO:23, hybridizes preferentially to the wildtype sequence and is useful as a control sequence. 5'CAG AAG CAG GTA GAA AT3', SEQ ID NO:24, is designed to hybridize preferentially to the mutant sequence.

The primers of the invention embrace oligonucleotides of sufficient length and appropriate sequence to provide initiation of polymerization on a significant number of nucleic acids in the polymorphic locus.

Preferred sequences for the present invention are SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:9, SEQ

ID NO:10, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:21, and SEQ ID NO:22. Environmental conditions conducive to synthesis of extension products include the presence of nucleoside triphosphates, an agent for polymerization, such as DNA polymerase, and suitable conditions such as temperature, ionic strength and pH. The primer is preferably single stranded for maximum efficiency in amplification, but may be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent for polymerization. The exact length of primer will depend on many factors, including temperature, buffer, and nucleotide composition. The oligonucleotide primer typically contains 12-20 or more nucleotides, although it may contain fewer nucleotides.

Primers of the invention are designed to be "substantially" complementary to each strand of the genomic locus to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands under conditions which allow the agent for polymerization to perform. In other words, the primers should have sufficient complementarity with the 5' and 3' sequences flanking the mutation to hybridize therewith and permit amplification of the genomic locus.

Oligonucleotide primers of the invention are employed in the amplification process which is an enzymatic chain reaction that produces exponential quantities of polymorphic locus relative to the number of reaction steps involved. Typically, one primer is complementary to the negative (-) strand of the

polymorphic locus and the other is complementary to the positive (+) strand. Annealing the primers to denatured nucleic acid followed by extension with an enzyme, such as the large fragment of DNA polymerase I (Klenow) and nucleotides, results in newly synthesized + and - strands containing the target polymorphic locus sequence. Because these newly synthesized sequences are also templates, repeated cycles of denaturing, primer annealing, and extension results in exponential production of the region (*i.e.*, the target polymorphic locus sequence) defined by the primers. The product of the chain reaction is a discreet nucleic acid duplex with termini corresponding to the ends of the specific primers employed.

The oligonucleotide primers of the invention may be prepared using any suitable method, such as conventional phosphotriester and phosphodiester methods or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage, et al., Tetrahedron Letters, 22:1859-1862, (1981). One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Patent No. 4,458,066.

Any nucleic acid specimen, in purified or nonpurified form, can be utilized as the starting nucleic acid or acids, providing it contains, or is suspected of containing, the specific nucleic acid sequence containing the polymorphic locus. Thus, the process may amplify, for example, DNA or RNA, including messenger RNA, wherein DNA or RNA may be single stranded or double stranded. In the event that RNA is to be used as a template, enzymes, and/or conditions optimal for reverse transcribing the template to DNA would be utilized.

In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of nucleic acids may also be employed, or the nucleic acids produced in a previous amplification reaction herein, using the same or different primers may be so utilized. The specific nucleic acid sequence to be amplified, *i.e.*, the polymorphic locus, may be a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor fraction of a complex mixture, such as contained in whole human DNA.

DNA utilized herein may be extracted from a body sample, such as blood, tissue material and the like by a variety of techniques such as that described by Maniatis, *et. al.* in *Molecular Cloning:A Laboratory Manual,* Cold Spring Harbor, NY, p 280-281, 1982). If the extracted sample is impure, it may be treated before amplification with an amount of a reagent effective to open the cells, or animal cell membranes of the sample, and to expose and/or separate the strand(s) of the nucleic acid(s). This lysing and nucleic acid denaturing step to expose and separate the strands will allow amplification to occur much more readily.

The deoxyribonucleotide triphosphates dATP, dCTP, dGTP, and dTTP are added to the synthesis mixture, either separately or together with the primers, in adequate amounts and the resulting solution is heated to about 90°-100°C from about 1 to 10 minutes, preferably from 1 to 4 minutes. This is sufficient to denature any double strands. After this heating period, the solution is allowed to cool at a rate which is preferable for the primer hybridization. To the cooled

mixture is added an appropriate agent for effecting the primer extension reaction (called herein "agent for polymerization"), and the reaction is allowed to occur under conditions known in the art. The agent for polymerization may also be added together with the other reagents if it is heat stable. This synthesis (or amplification) reaction may occur at room temperature up to a temperature above which the agent for polymerization no longer functions. Thus, for example, if DNA polymerase is used as the agent, the temperature is generally no greater than about 40°C. Thermostable DNA polymerases, such as Taq polymerase may function at a higher temperature.

The agent for polymerization may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase, polymerase muteins, reverse transcriptase, other enzymes, including heat-stable enzymes (*i.e.*, those enzymes which perform primer extension after being subjected to temperatures sufficiently elevated to cause denaturation), such as *Taq* polymerase. The suitable enzyme will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each polymorphic locus nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths.

The newly synthesized strand and its complementary nucleic acid strand will form a double-stranded molecule under hybridizing conditions described above and this hybrid is used in subsequent steps of the process. In the next step, the

newly synthesized double-stranded molecule is subjected to denaturing conditions using any of the procedures described above to provide single-stranded molecules.

The steps of denaturing, annealing, and extension product synthesis can be repeated as often as needed to amplify the target polymorphic locus nucleic acid sequence to the extent necessary for detection. The amount of the specific nucleic acid sequence produced will accumulate in an exponential fashion. PCR. A Practical Approach, ILR Press, Eds. M. J. McPherson, P. Quirke, and G. R. Taylor, 1992.

The amplification products may be detected by analyzing it by Southern blots without using radioactive probes. In such a process, for example, a small sample of DNA containing a very low level of the nucleic acid sequence of the polymorphic locus is amplified, and analyzed via a Southern blotting technique or similarly, using dot blot analysis. The use of non-radioactive probes or labels is facilitated by the high level of the amplified signal. Alternatively, probes used to detect the amplified products can be directly or indirectly detectably labeled, for example, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Those of ordinary skill in the art will know of other suitable labels for binding to the probe, or will be able to ascertain such, using routine experimentation. In the preferred embodiment, the amplification products are determinable by separating the mixture on an agarose gel containing ethidium bromide which causes DNA to be fluorescent.

Sequences amplified by the methods of the invention can be further evaluated, detected, cloned, sequenced, and the

like, either in solution or after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence such as PCR, oligomer restriction (Saiki, *et.al.*, *Bio/Technology*, 3:1008-1012, 1985), allele-specific oligonucleotide (ASO) probe analysis (Conner, *et. al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 80:278, 1983), oligonucleotide ligation assays (OLAs) (Landgren, *et. al.*, *Science*, 241:1007, 1988), and the like. Molecular techniques for DNA analysis have been reviewed (Landgren, *et. al.*, *Science*, 242:229-237, 1988).

Preferably, the method of amplifying is by PCR, as described herein and as is commonly used by those of ordinary skill in the art. Alternative methods of amplification have been described and can also be employed as long as the BRCA2 locus amplified by PCR using primers of the invention is similarly amplified by the alternative means. Such alternative amplification systems include but are not limited to self-sustained sequence replication, which begins with a short sequence of RNA of interest and a T7 promoter. Reverse transcriptase copies the RNA into cDNA and degrades the RNA, followed by reverse transcriptase polymerizing a second strand of DNA. Another nucleic acid amplification technique is nucleic acid sequence-based amplification (NASBA) which uses reverse transcription and T7 RNA polymerase and incorporates two primers to target its cycling scheme. NASBA can begin with either DNA or RNA and finish with either, and amplifies to 10^8 copies within 60 to 90 minutes. Alternatively, nucleic acid can be amplified by ligation activated transcription (LAT). LAT works from a single-stranded template with a single primer that is partially single-stranded and partially double-stranded. Amplification is initiated by ligating a

cDNA to the promoter oligonucleotide and within a few hours, amplification is 10^8 to 10^9 fold. The QB replicase system can be utilized by attaching an RNA sequence called MDV-1 to RNA complementary to a DNA sequence of interest. Upon mixing with a sample, the hybrid RNA finds its complement among the specimen's mRNAs and binds, activating the replicase to copy the tag-along sequence of interest. Another nucleic acid amplification technique, ligase chain reaction (LCR), works by using two differently labeled halves of a sequence of interest which are covalently bonded by ligase in the presence of the contiguous sequence in a sample, forming a new target. The repair chain reaction (RCR) nucleic acid amplification technique uses two complementary and target-specific oligonucleotide probe pairs, thermostable polymerase and ligase, and DNA nucleotides to geometrically amplify targeted sequences. A 2-base gap separates the oligonucleotide probe pairs, and the RCR fills and joins the gap, mimicking normal DNA repair. Nucleic acid amplification by strand displacement activation (SDA) utilizes a short primer containing a recognition site for *Hinc II* with short overhang on the 5' end which binds to target DNA. A DNA polymerase fills in the part of the primer opposite the overhang with sulfur-containing adenine analogs. *Hinc II* is added but only cuts the unmodified DNA strand. A DNA polymerase that lacks 5' exonuclease activity enters at the cite of the nick and begins to polymerize, displacing the initial primer strand downstream and building a new one which serves as more primer. SDA produces greater than 10^7-fold amplification in 2 hours at 37°C. Unlike PCR and LCR, SDA does not require instrumented Temperature cycling. Another amplification system useful in the method of the invention is the QB Replicase System.

Although PCR is the preferred method of amplification in the invention, these other methods can also be used to amplify the BRCA2 locus as described in the method of the invention.

In another embodiment of the invention, a method is provided for diagnosing a subject having a predisposition or higher susceptibility to (at risk of) breast or ovarian cancer comprising sequencing a target nucleic acid of a sample from a subject by dideoxy sequencing following amplification of the target nucleic acid. In such an embodiment, one does not even need to use any of the oligonucleotides, either primers or probes, described herein. The BRCA2 gene, or fragments thereof, may be directly cloned and then sequenced (such as by dideoxy methods) to determine the presence or absence of a mutation. In such a situation, one need only compare the sequence obtained to a naturally occurring (wild type) BRCA2 gene, or portion thereof.

Other methods of DNA sequencing such as those of Sanger et al, Proc. Natl. Acad. Sci. USA, 74: 5463 (1977) or Maxam et al, Proc. Natl. Acad. Sci. USA, 74: 560 (1977) or other methods known in the art may be used.

In another embodiment of the invention a method is provided for diagnosing a subject having a predisposition or higher susceptibility to (at risk of) breast or ovarian cancer comprising contacting a target nucleic acid of a sample from a subject with a reagent that detects the presence of one of the mutations of the present invention and detecting the mutation.

In another embodiment of the invention, a method and reagents are provided for repairing the gene mutation in at least some cells by applying an oligomer comprising the sequence of the wild-type probes to repair the individual's genome by triple strand hybridization. See U.S. Patents

5,650,316 and 5,624,803 for example. This is a form of gene therapy to correct the defect in either apparently normal tissue or in an active tumor. Gene repair may also be performed on excized tumor cells which may be helpful in determining the preferred therapy to be used, particularly the reagents used for gene therapy. Other forms of gene therapy, such as providing a complete copy of a normal BRCA2 gene may also be used.

In another embodiment of the invention a method is provided for characterizing a tumor. Histologic type, morphologic grade, differences between inherited and sporadic breast cancer do not appear to be distinguished. One method comprises sequencing the target nucleic acid isolated from the tumor or other biological sample to determine if the mutation is has occured or is present. Sanger, F., *et al.*, *J. Mol. Biol.*, 142:1617 (1980).

Characterizing a tumor as having originated from an inherited breast cancer gene may be clinically significant as the prevalence of bilateral breast cancer is higher than in sproadic cases. Weber, Scientific American, JAN-FEB p. 12-21 (1996). The tumor may be classified based on tissue taken from the tumor itself or from a non-tumor site which contains genomic DNA.

Yet another embodiment of the present invention is an isolated mutant BRCA2 DNA sequence which may be the entire sequence, an exon thereof or a fragment thereof. The DNA sequence must contain at least one mutation from the list: C2192G, 3772delTT, C5193G, 5374del4, 6495delGC or 6909insG. Preferably, the isolated DNA sequence contains a sequence complementary to at least one of the following: SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:12:, SEQ ID NO:16, SEQ ID NO:20, or SEQ

ID NO:24. This sequence has usefulness alone, or after cloning and expression to determine suitable treatments to prevent formation of a tumor, prevent transmission of the mutant gene to offspring or to decide other prophylactic diagnostic and treatment protocols. The isolated DNA sequence may also be used for drug design by protein replacement, protein mimetics, screaning known and unknown compounds, anti-idiotype antibodies to the BRCA1 active site for the preparation of an immunogen or vaccine and determining appropriate gene therapy to counter the pathology associated with the mutant BRCA2 gene. For diagnostic purposes, knowing the mutant BRCA2 sequence for comparison purposes is the critical step in diagnosis.

Another method comprises contacting a target nucleic acid of a sample from a subject with a reagent that detects the presence of the mutation and detecting the mutation. A number of hybridization methods are well known to those skilled in the art. Many of them are useful in carrying out the invention.

The materials for use in the method of the invention are ideally suited for the preparation of a diagnostic kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, tubes, and the like, each of the container means comprising one or more of the separate elements to be used in the method. For example, one of the container means may comprise means for amplifying BRCA2 DNA, said means comprising the necessary enzyme(s) and oligonucleotide primers for amplifying said target DNA from the subject. Another container may contain oligonucleotide probes for detecting the presence or absence of a mutation.

The oligonucleotide primers include primers having a sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:21, SEQ ID NO:22 or primer sequences substantially complementary or substantially homologous thereto. Other primers flanking the BRCA2 locus or a region containing one of the mutation sites may be used. The target flanking 5' and 3' polynucleotide sequence include other oligonucleotide primers for amplifying the BRCA2 locus will be known or readily ascertainable to those of skill in the art.

Oligonucleotide probes including probes having substantially the sequence of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:23, SEQ ID NO:24. Other oligonucleotide probes which hybridize to one or more of the BRCA2 mutation sites and sequences substantially complementary or homologous thereto may be used. Other oligonucleotide probes for detecting the mutations will be known or readily ascertainable to those of skill in the art.

The following definitions are provided for the purpose of understanding this invention.

The term "primer" as used herein refers to a sequence comprising two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and more preferably more than eight and most preferably at least 20 nucleotides of the BRCA2 gene wherein the sequence corresponds to a sequence flanking one of the mutations or wild type sequences of BRCA2 corresponding to the mutation sites. Primers may be used to initiate DNA synthesis via the PCR.

The primers of the present invention include the sequences recited and complementary sequences which would aneal to the opposite DNA strand of the sample target. Since both strands of DNA are complementary and mirror immages of each other, the same segment of DNA will be amplified.

The term "substantially complementary to" or "substantially the sequence" refers to sequences which hybridize to the sequences provided under stringent conditions and/or sequences having sufficient homology with, (e.g. SEQ ID NO:3 and SEQ ID NO:4) such that the allele specific oligonucleotides of the invention hybridize to the sequence. "Substantially" the same as it referrs to oligonucleotide sequences also referrs to the functional ability to hybridize or aneal with sufficient specificity to distinguish between the presence or absence of the mutation. This is measurable by the temperature of melting being sufficiently different to permit easy identification of whether the oligonucleotide is binding to the normal or mutant BRCA2 gene sequence.

The term "isolated" as used herein refers to being substantially free of other polynucleic acids, proteins, lipids, carbohydrates or other materials with which they may be associated. Such association being either in cellular material or in a synthesis medium.

"Biological sample" referrs to a polynucleotide containing sample originally from a biological source. The sample may be from a living, dead or even archeological source from a variety of tissues and cells. Examples include: body fluid [blood (leukocytes), urine (epithelial cells), saliva, cervical and vaginal secretions...] skin, hair roots/folicle, mucus membrane (e.g. buccal or tongue cell scrapings), cervicovaginal cells (from PAP smear, etc.) internal tissue

(normal or tumor), chorionic villus tissue, amnionic cells, placental cells, fetal cells, cord blood, sperm or egg.

"Coding sequence" or "DNA coding sequence" refers to those portions of a gene which, taken together, code for a peptide (protein), or for which the nucleic acid itself has function.

A "target polynucleotide" refers to the nucleic acid sequence of interest *e.g.*, the BRCA2 encoding polynucleotide.

"Consensus" means the most commonly occurring in the population.

"Cancer", "tumor" and other similar terms refer to any neoplasm whether benign or malignant, and regardless of whether it has metastisized or the location of the "cancer" or "tumor".

"Substantially complementary to" refers to probe or primer sequences which hybridize to the sequences listed under stringent conditions and/or sequences having sufficient homology with test polynucleotide sequences, such that the allele specific oligonucleotide probe or primers hybridize to the test polynucleotide sequences to which they are complimentary.

"Sequence variation" as used herein refers to any difference in nucleotide sequence between two different oligonucleotide or polynucleotide sequences.

"Polymorphism" as used herein refers to a sequence variation in a gene which is not necessarily associated with pathology.

"Mutation" as used herein refers to an altered genetic sequence which results in the gene coding for a non-functioning protein or a protein with substantially reduced or

altered function. Generally, a deleterious mutation is associated with pathology or the potential for pathology.

"Pre-determined sequence variation" as used herein refers to a nucleotide sequence that is designed to be different than the corresponding sequence in a reference nucleotide sequence. A pre-determined sequence variation can be a known mutation in the BRCA2 gene.

"BRCA2 gene" is a group of compounds and refers to the published gene sequences, those appearing in the GENBANK database and the BIC database. Other different sequences include polymorphisms and genetic alterations, especially those which define other haplotypes for the BRCA2 gene. Generally polymorphisms which don't cause an amino acid change or which are naturally occurring (wild types), which are not associated with pathology are also considered the BRCA2 gene. The corresponding nucleotides would then be used even if the nucleotide number differs. While the BRCA2 gene discussed herein is the human BRCA2 gene, the corresponding assays and reagents for the gene in other animals may also be used. The BRCA2 gene includes the coding sequences, non-coding sequences (e.g. introns) and regulatory regions affecting gene expression.

"Allele specific detection assay" as used herein refers to an assay to detect the presence or absence of a predetermined sequence variation in a test polynucleotide or oligonucleotide by annealing the test polynucleotide or oligonucleotide with a polynucleotide or oligonucleotide of predetermined sequence such that differential DNA sequence based techniques or DNA amplification methods discriminate between normal and mutant.

"Sequence variation locating assay" as used herein refers to an assay that detects a sequence variation in a test polynucleotide or oligonucleotide and localizes the position of the sequence variation to a subregion of the test polynucleotide, without necessarily determining the precise base change or position of the sequence variation.

"Region" as used herein generally refers to an area from several nucleotides upstream to several nucleotides downstream from the specific nucleotide mentioned. "Region" also includes the complementary nucleotides on the antisense strand of sample DNA.

"Targeted confirmatory sequencing" as used herein refers to sequencing a polynucleotide in the region wherein a sequence variation has been located by a sequence variation locating assay in order to determine the precise base change and/or position of the sequence variation.

"Probe" includes any oligonucleotide which hybridizes to a BRCA2 or mutant BRCA2 sequence. The probe may be labled (directly or indirectly) or it may act as a primer such as a PCR primer. The probes of the present invention include the sequences recited and complementary sequences which would aneal to the antisense strand of the sample target DNA. Since both strands of DNA are complementary and mirror immages of each other, the complementary version of the mutation is equally unique and indicative of the mutation to be assayed.

Allele Specific Oligonucleotide hybridization is sometimes referred to ASO or the ASO method.

The invention in several of its embodiments includes:

DETECTION OF PRE-DETERMINED SEQUENCE VARIATIONS

Stage I analysis may be used to determine the presence or absence of a pre-determined nucleotide sequence variation; preferably a known mutation or set of known mutations in the test gene. In accordance with the invention, such pre-determined sequence variations are detected by allele specific hybridization, a sequence-dependent-based technique which permits discrimination between normal and mutant alleles. An allele specific assay is dependent on the differential ability of mismatched nucleotide sequences (*e.g.*, normal:mutant) to hybridize with each other, as compared with matching (*e.g.*, normal:normal or mutant:mutant) sequences.

DETECTION OF PRE-DETERMINED SEQUENCE VARIATIONS USING ALLELE SPECIFIC HYBRIDIZATION

A variety of methods well-known in the art can be used for detection of pre-determined sequence variations by allele specific hybridization. Preferably, the test gene is probed with allele specific oligonucleotides (ASOs); and each ASO contains the sequence of a known mutation. ASO analysis detects specific sequence variations in a target polynucleotide fragment by testing the ability of a specific oligonucleotide probe to hybridize to the target polynucleotide fragment. Preferably, the oligonucleotide contains the mutant sequence (or its complement). The presence of a sequence variation in the target sequence is indicated by hybridization between the oligonucleotide probe and the target fragment under conditions in which an oligonucleotide probe containing a normal sequence does not hybridize to the target fragment. A lack of hybridization between the sequence variant (*e.g.*, mutant) oligonucleotide probe and the target

polynucleotide fragment indicates the absence of the specific sequence variation (*e.g.*, mutation) in the target fragment. In a preferred embodiment, the test samples are probed in a standard dot blot format. Each region within the test gene that contains the sequence corresponding to the ASO is individually applied to a solid surface, for example, as an individual dot on a membrane. Each individual region can be produced, for example, as a separate PCR amplification product using methods well-known in the art (see, for example, the experimental embodiment set forth in Mullis, K.B., 1987, U.S. Patent No. 4,683,202). The use of such a dot blot format is described in detail in the Examples below, detailing the Stage I analysis of the human BRCA2 gene to detect the presence or absence of six different known mutations using six corresponding ASOs.

Membrane-based formats that can be used as alternatives to the dot blot format for performing ASO analysis include, but are not limited to, reverse dot blot, (multiplex amplification assay), and multiplex allele-specific diagnostic assay (MASDA).

In a reverse dot blot format, oligonucleotide or polynucleotide probes having known sequence are immobilized on the solid surface, and are subsequently hybridized with the labeled test polynucleotide sample. In this situation, the primers may be labeled or the NTPs maybe labeled prior to amplification to prepare a labeled test polynucleotide sample. Alternatively, the test polynucleotide sample may be labeled subsequent to isolation and/or synthesis.

In a multiplex format, individual samples contain multiple target sequences within the test gene, instead of just a single target sequence. For example, multiple PCR

products each containing at least one of the ASO target sequences are applied within the same sample dot. Multiple PCR products can be produced simultaneously in a single amplification reaction using the methods of Caskey et al., U.S. Patent No. 5,582,989. The same blot, therefore, can be probed by each ASO whose corresponding sequence is represented in the sample dots.

A MASDA format expands the level of complexity of the multiplex format by using multiple ASOs to probe each blot (containing dots with multiple target sequences). This procedure is described in detail in U.S. Patent No. 5,589,330 by A.P. Shuber, and in Michalowsky et al., American Journal of Human Genetics, 59(4): A272, poster 1573, October 1996, each of which is incorporated herein by reference in its entirety. First, hybridization between the multiple ASO probe and immobilized sample is detected. This method relies on the prediction that the presence of a mutation among the multiple target sequences in a given dot is sufficiently rare that any positive hybridization signal results from a single ASO within the probe mixture hybridizing with the corresponding mutant target. The hybridizing ASO is then identified by isolating it from the site of hybridization and determining its nucleotide sequence.

Suitable materials that can be used in the dot blot, reverse dot blot, multiplex, and MASDA formats are well-known in the art and include, but are not limited to nylon and nitrocellulose membranes.

When the target sequences are produced by PCR amplification, the starting material can be chromosomal DNA in which case the DNA is directly amplified. Alternatively, the starting material can be mRNA, in which case the mRNA is first

reversed transcribed into cDNA and then amplified according to the well known technique of RT-PCR (see, for example, U.S. Patent No. 5,561,058 by Gelfand et al.).

The methods described above are suitable for moderate screening of a limited number of sequence variations. However, with the need in molecular diagnosis for rapid, cost effective large scale screening, technologies have developed that integrate the basic concept of ASO, but far exceed the capacity for mutation detection and sample number. These alternative methods to the ones described above include, but are not limited to, large scale chip array sequence-based techniques. The use of large scale arrays allows for the rapid analysis of many sequence variants. A review of the differences in the application and development of chip arrays is covered by Southern, E.M., Trends In Genetics, 12: 110-115 (March 1996) and Cheng et al., Molecular Diagnosis, 1:183-200 (September 1996). Several approaches exist involving the manufacture of chip arrays. Differences include, but not restricted to: type of solid support to attach the immobilized oligonucleotides, labeling techniques for identification of variants and changes in the sequence-based techniques of the target polynucleotide to the probe.

A promising methodology for large scale analysis on 'DNA chips' is described in detail in Hacia et al., Nature Genetics, 14:441-447, (1996) which is hereby incorporated by reference in its entirety. As described in Hacia et al., high density arrays of over 96,000 oligonucleotides, each 20 nucleotides in length, are immobilized to a single glass or silicon chip using light directed chemical synthesis. Contingent on the number and design of the oligonucleotide probe, potentially every base in a sequence can be

interrogated for alterations. Oligonucleotides applied to the chip, therefore, can contain sequence variations that are not yet known to occur in the population, or they can be limited to mutations that are known to occur in the population.

Prior to hybridization with olignucleotide probes on the chip, the test sample is isolated, amplified and labeled (e.g. fluorescent markers) by means well known to those skilled in the art. The test polynucleotide sample is then hybridized to the immobilized oligonucleotides. The intensity of sequence-based techniques of the target polynucleotide to the immobilized probe is quantitated and compared to a reference sequence. The resulting genetic information can be used in molecular diagnosis.

A common, but not limiting, utility of the 'DNA chip' in molecular diagnosis is screening for known mutations. However, this may impose a limitation on the technique by only looking at mutations that have been described in the field. The present invention allows allele specific hybridization analysis be performed with a far greater number of mutations than previously available. Thus, the efficiency and comprehensiveness of large scale ASO analysis will be broadened, reducing the need for cumbersome end-to-end sequence analysis, not only with known mutations but in a comprehensive manner all mutations which might occur as predicted by the principles accepted, and the cost and time associated with these cumbersome tests will be decreased.

EXAMPLE

Genomic DNA (at least 100 ng) is isolated from white blood cells of a subject with a family history of breast, ovarian or other cancer. Dideoxy sequence analysis is

performed following polymerase chain reaction amplification of segments of exon 11.

Exon 11 of the BRCA2 gene is subjected to direct dideoxy sequence analysis by asymmetric amplification using the polymerase chain reaction (PCR) to generate a single stranded product amplified from this DNA sample. Shuldiner, *et al.*, Handbook of Techniques in Endocrine Research, p. 457-486, DePablo,F., Scanes, C., eds., Academic Press, Inc., 1993. Fluorescent dye is attached for automated sequencing using the TAQ DYE TERMINATOR KIT (PERKIN-ELMER cat# 401628). DNA sequencing is performed in both forward and reverse directions on an APPLIED BIOSYSTEMS, INC. (ABI) automated sequencer (Model 373 or 377). The software used for analysis of the resulting data is "SEQUENCE NAVIGATOR" purchased through ABI.

The methods of the invention, which can be used to detect sequence variations in any polynucleotide sample, are demonstrated in the Example set forth in this section, for the purpose of illustration, for one gene in particular, namely, the human BRCA2 gene. The BRCA2 coding sequence is approximately 10,248 base pairs encoding the 3418 amino acid BRCA2 protein.

Designing an Allele Specific Oligonucleotide (ASO) Probe

An allele specific oligonucleotide probe is a short, single stranded polynucleotide that is engineered to hybridize exactly to a target sequence under a given set of conditions. Routinely, ASO probes are designed to contain sequences identical to the normal allele and sequence variation respectively. Hybridization of the probe to the target allows for the discrimination of a variant sample. Under stringent

conditions, a probe with a variation as simple as a single-base pair will not hybridize to a normal sequence due to a destabilizing effect of the normal-mutant duplex (Ikuta, S. et al, Nucleic Acids Research, 15: 797-811 (1987). For use in this invention, probes were used to discriminate between a wild-type or normal sequence from one that is mutated. Each probe pair contained a polynucleotide sequence that encompassed an area that would identify a selected mutation of the BRCA 2 gene.

The design of an ASO hybridization probe must meet two basic requirements. (Current Protocols in Human Genetics, section 9.4, (1995)). First, probes that are used together in the same pool should be around the same length. Although the standard length of a probe is optimally 17 base pairs, the range can be as short as about 14 or as long as about 24. Second, the mismatched region should not be placed at the end of the 17 base pair probe, but approximately in the middle of the sequence, approximately 5-7 bases from the 5' end of the probe. In addition, the placement of a mismatch, in the case of a longer probe, should not be at the end, but at a position that allows strong hybridization and stabilization of the polynucleotide strand. In order to minimize the effects of variations in base composition of the probes, tetramethylammonium chloride is used as in the ASO hybrid's buffer (Shuber, T., US Patent # 5,633,134). Conventionally, ASO probes are synthesized on a DNA synthesizer. They can be labeled with isotopic or non-isotopic detection agents using means familiar to those of skill in the art. The process outlined in this application for making and using probes can be applicable for other gene sequences.

DETAILED METHOD FOR THE DETECTION OF SEQUENCE VARIATIONS IN POLYNUCLEOTIDES

Isolation of Genomic DNA

White blood cells were collected from the patient and genomic DNA is extracted from the white blood cells according to well-known methods (Sambrook, et al., Molecular Cloning, A Laboratory Manual, 2nd Ed., 1989, Cold Spring Harbor Laboratory Press, at 9.16 - 9.19).

PCR Amplification for Sequencing

The genomic DNA is used as a template to amplify a separate DNA fragment encompassing the site of the mutation to be tested. The 25 μl PCR reaction contained the following components: 1 μl template (100 ng/ μl) DNA, 2.5 μl 10X PCR Buffer (PERKIN-ELMER), 1.5 μl dNTP (2mM each dATP, dCTP, dGTP, dTTP), 1.5 μl Forward Primer (10 μM), 1.5 μl Reverse Primer (10 μM), 0.5 μl (2.5U total) AMPLITAQ GOLD™ TAQ DNA POLYMERASE or AMPLITAQ® TAQ DNA POLYMERASE (PERKIN-ELMER), 1.0 to 5.0 μl (25mM) $MgCl_2$ (depending on the primer) and distilled water (dH_2O) up to 25 μl. All reagents for each exon except the genomic DNA can be combined in a master mix and aliquoted into the reaction tubes as a pooled mixture.

For each exon analyzed, the following control PCRs were set up:

(1) "Negative" DNA control (100 ng placental DNA (SIGMA CHEMICAL CO., St. Louis, MO)

(2) Three "no template" controls

PCR for all exons is performed using the following thermocycling conditions:

Temperature	Time	Number of Cycles
95°C	5 min. (AMPLITAQ) or 10 min. (GOLD)	1
95°C	30 sec.	30 cycles
55°C	30 sec.	
72°C	1 min	
72°C	5 min.	1
4°C	infinity	1

Quality control agarose gel of PCR amplification:

The quality of the PCR products were examined prior to further analysis by electrophoresing an aliquot of each PCR reaction sample on an agarose gel. 5μl of each PCR reaction is run on an agarose gel along side a DNA 100BP DNA LADDER (Gibco BRL cat# 15628-019). The electrophoresed PCR products were analyzed according to the following criteria:

Each patient sample must show a single band of the size corresponding the number of base pairs expected from the length of the PCR product from the forward primer to the reverse primer. If a patient sample demonstrates smearing or multiple bands, the PCR reaction must be repeated until a clean, single band is detected. If no PCR product is visible or if only a weak band is visible, but the control reactions with placental DNA template produced a robust band, the patient sample should be re-amplified with 2X as much template DNA.

All three "no template" reactions must show no amplification products. Any PCR product present in these reactions is the result of contamination. If any one of the "no template" reactions shows contamination, all PCR products should be discarded and the entire PCR set of reactions should be repeated after the appropriate PCR decontamination procedures have been taken.

The optimum amount of PCR product on the gel should be between 50 and 100 ng, which can be determined by comparing the intensity of the patient sample PCR products with that of the DNA ladder. If the patient sample PCR products contain less than 50 to 100 ng, the PCR reaction should be repeated until sufficient quantity is obtained.

DNA Sequencing

For DNA sequencing, double stranded PCR products are labeled with four different fluorescent dyes, one specific for each nucleotide, in a cycle sequencing reaction. With Dye Terminator Chemistry, when one of these nucleotides is incorporated into
the elongating sequence it causes a termination at that point. Over the course of the cycle sequencing reaction, the dye-labeled nucleotides are incorporated along the length of the PCR product generating many different length fragments.

The dye-labeled PCR products will separate according to size when electrophoresed through a polyacrylamide gel. At the lower portion of the gel on an ABI automated sequencers, the fragments pass through a region where a laser beam continuously scans across the gel. The laser excites the fluorescent dyes attached to the fragments causing the emission of light at a specific wavelength for each dye. Either a photomultiplier tube (PMT) detects the fluorescent light and converts is into an electrical signal (ABI 373) or the light is collected and separated according to wavelength by a spectrograph onto a cooled, charge coupled device (CCD) camera (ABI 377). In either case the data collection software will collect the signals and store them for subsequent sequence analysis.

PCR products were first purified for sequencing using a QIAQUICK-SPIN PCR PURIFICATION KIT (QIAGEN #28104). The purified PCR products were labeled by adding primers, fluorescently tagged dNTPs and Taq Polymerase FS in an ABI Prism Dye Terminator Cycle Sequencing Kit (PERKIN ELMER/ABI catalog #02154) in a PERKIN ELMER GENEAMP 9600 thermocycler.

The amounts of each component are:

For Samples		For Controls	
Reagent	Volume	Reagent	Volume
Dye mix	8.0 μL	PGEM	2.0 μL
Primer (1.6mM)	2.0 μL	M13	2.0 μL
PCR product	2.0 μL	Dye mix	8.0 μL
sdH2O	8.0 μL	sdH2O	8.0 μL

The thermocycling conditions were:

Temperature	Time	# of Cycles
96°C	15 sec.	\
50°C	5 sec.	} 25
60°C	4 min.	/
4°C	Infinity	1

The product was then loaded into a gel and placed into an ABI DNA Sequencer (Models 373A & 377) and run. The sequence obtained was analyzed by comparison to the wild type (reference) sequence within the SEQUENCE NAVIGATOR. When a sequence does not align, it indicates a possible mutation. The DNA sequence was determined in both the forward and reverse direction. All results were provided to a second reader for review.

Heterozygous/homozygous point mutations and polymorphisms must be seen in both strands. Frameshift mutations will be seen in both strands and must have clear double peaks in frame shift regions to be so identified.

PCR Amplification for ASO

The genomic DNA is used as a template to amplify a separate DNA fragment encompassing the site of the mutation to be tested. The 50 μl PCR reaction contained the following components: 1 μl template (100 ng/ μl) DNA, 5.0 μl 10X PCR Buffer (PERKIN-ELMER), 2.5 μl dNTP (2mM each dATP, dCTP, dGTP, dTTP), 2.5 μl Forward Primer (10 μM), 2.5 μl Reverse Primer (10 μM), 0.5 μl (2.5U total) AMPLITAQ® TAQ DNA POLYMERASE or AMPLITAQ GOLD™ DNA POLYMERASE (PERKIN-ELMER), 1.0 to 5.0 μl (25mM) $MgCl_2$ (depending on the primer) and distilled water (dH_2O) up to 50 μl. All reagents for each exon except the genomic DNA can be combined in a master mix and aliquoted into the reaction tubes as a pooled mixture.

For each exon analyzed, the following control PCRs were set up:

(1) "Negative" DNA control (100 ng placental DNA (SIGMA CHEMICAL CO., St. Louis, MO)

(2) Three "no template" controls

PCR for all exons is performed using the following thermocycling conditions:

Temperature	Time	Number of Cycles
95°C	5 min. (AMPLITAQ) or 10 min. (GOLD)	1
95°C	30 sec.	30 cycles
55°C	30 sec.	
72°C	1 min	
72°C	5 min.	1
4°C	infinity	1

The quality control agarose gel of PCR amplification was performed as above.

Binding PCR Products to Nylon Membrane

The PCR products are denatured no more than 30 minutes prior to binding the PCR products to the nylon membrane. To denature the PCR products, the remaining PCR reaction (45 μl) and the appropriate positive control mutant gene amplification product are diluted to 200 μl final volume with PCR Diluent Solution (500 mM NaOH, 2.0 M NaCl, 25 mM EDTA) and mixed thoroughly. The mixture is heated to 95°C for 5 minutes, and immediately placed on ice and held on ice until loaded onto dot blotter, as described below.

The PCR products are bound to 9 cm by 13 cm nylon ZETA PROBE BLOTTING MEMBRANE (BIO-RAD, Hercules, CA, catalog number 162-0153) using a BIO-RAD dot blotter apparatus. Forceps and gloves are used at all times throughout the ASO analysis to manipulate the membrane, with care taken never to touch the surface of the membrane with bare hands or latex gloves.

Pieces of 3MM filter paper [WHATMAN®, Clifton, NJ] and nylon membrane are pre-wet in 10X SSC prepared fresh from 20X SSC buffer stock. The vacuum apparatus is rinsed thoroughly with dH_2O prior to assembly with the membrane. 100 μl of each denatured PCR product is added to the wells of the blotting apparatus. Each row of the blotting apparatus contains a set of reactions for a single exon to be tested, including a placental DNA (negative) control, a synthetic oligonucleotide with the desired mutation or a PCR product from a known mutant sample (positive control), and three no template DNA controls.

After applying PCR products, the nylon filter is placed DNA side up on a piece of 3MM filter paper saturated with denaturing solution (1.5M NaCl, 0.5 M NaOH) for 5 minutes. The membrane is transferred to a piece of 3MM filter paper saturated with neutralizing solution (1M Tris-HCl, pH 8, 1.5 M

NaCl) for 5 minutes. The neutralized membrane is then transferred to a dry 3MM filter DNA side up, and exposed to ultraviolet light (STRALINKER, STRATAGENE, La Jolla, CA) for exactly 45 seconds the fix the DNA to the membrane. This UV crosslinking should be performed within 30 min. of the denaturation/neutralization steps. The nylon membrane is then cut into strips such that each strip contains a single row of blots of one set of reactions for a single exon.

Hybridizing Labeled Oligonucleotides to the Nylon Membrane

Prehybridization

The strip is prehybridized at 52°C using the HYBAID® (SAVANT INSTRUMENTS, INC., Holbrook, NY) hybridization oven. 2X SSC (15 to 20 ml) is preheated to 52°C in a water bath. For each nylon strip, a single piece of nylon mesh cut slightly larger than the nylon membrane strip (approximately 1" x 5") is pre-wet with 2X SSC. Each single nylon membrane is removed from the prehybridization solution and placed on top of the nylon mesh. The membrane/mesh "sandwich" is then transferred onto a piece of Parafilm. The membrane/mesh sandwich is rolled lengthwise and placed into an appropriate HYBAID® bottle, such that the rotary action of the HYBAID® apparatus caused the membrane to unroll. The bottle is capped and gently rolled to cause the membrane/mesh to unroll and to evenly distribute the 2X SSC, making sure that no air bubbles formed between the membrane and mesh or between the mesh and the side of the bottle. The 2X SSC is discarded and replaced with 5 ml TMAC Hybridization Solution, which contained 3 M TMAC (tetramethyl ammoniumchloride - SIGMA T-3411), 100 mM Na_3PO_4(pH6.8), 1 mM EDTA, 5X Denhardt's (1% Ficoll, 1%

polyvinylpyrrolidone, 1% BSA (fraction V)), 0.6% SDS, and 100 μg/ml Herring Sperm DNA. The filter strips were prehybridized at 52°C with medium rotation (approx. 8.5 setting on the HYBAID® speed control) for at least one hour. Prehybridization can also be performed overnight.

Labeling Oligonucleotides

The DNA sequences of the oligonucleotide probes used to detect the BRCA2 mutations are as follows (for each mutation, a mutant and a normal oligonucleotide must be labeled):

C2192G - normal 5'TGA AGA ACC AAC TTT GT3' SEQ ID NO:3
C2192G - mutant 5'TGA AGA ACG AAC TTT GT3' SEQ ID NO:4
3772delTT - normal 5'GCA AGC AAT TTG AAG GT3' SEQ ID NO:7
3772delTT - mutant 5'GCA AGC AAT GAA GGT AC3' SEQ ID NO:8
C5193G - normal 5'ACT TGT TAC ACA AAT CA3' SEQ ID NO:11
C5193G - mutant 5'ACT TGT TAG ACA AAT CA3' SEQ ID NO:12
5374del4 - normal 5'ATT ATT TGT ATG AAA AT3' SEQ ID NO:15
5374del4 - mutant 5'ATT ATT TGA AAA TAA TT3' SEQ ID NO:16
6495delGC - normal 5'GAA CTG AGC ATA GTC TT3' SEQ ID NO:19
6495delGC - mutant 5'GAA CTG AAT AGT CTT CA3' SEQ ID NO:20
6909insG - normal 5'CAG AAG CAG TAG AAA TT3' SEQ ID NO:23
6909insG - mutant 5'CAG AAG CAG GTA GAA AT3' SEQ ID NO:24

Each labeling reaction contains 2-μl 5X Kinase buffer (or 1μl of 10X Kinase buffer), 5μl gamma-ATP ^{32}P (not more than one week old), 1μl T4 polynucleotide kinase, 3μl oligonucleotide (20 μM stock), sterile H_2O to 10 μl final volume if necessary. The reactions are incubated at 37°C for 30 minutes, then at 65°C for 10 minutes to heat inactivate the kinase. The kinase reaction is diluted with an equal volume (10μl) of sterile dH_2O (distilled water).

The oligonucleotides are purified on STE MICRO SELECT-D, G-25 spin columns (catalog no. 5303-356769), according to the manufacturer's instructions. The 20μl synthetic oligonucleotide eluate is diluted with 80 μl dH_20 (final volume = 100 μl). The amount of radioactivity in the oligonucleotide sample is determined by measuring the radioactive counts per minute (cpm). The total radioactivity must be at least 2 million cpm. For any samples containing less than 2 million total, the labeling reaction is repeated.

Hybridization with Mutant Oligonucleotides

Approximately 2-5 million counts of the labeled mutant oligonucleotide probe is diluted into 5 ml of TMAC hybridization solution, containing 40 μl of 20 μM stock of unlabeled normal oligonucleotide. The probe mix is preheated to 52°C in the hybridization oven. The pre-hybridization solution is removed from each bottle and replaced with the probe mix. The filter is hybridized for 1 hour at 52°C with moderate agitation. Following hybridization, the probe mix is decanted into a storage tube and stored at -20°C. The filter is rinsed by adding approximately 20 ml of 2x SSC + 0.1% SDS at room temperature and rolling the capped bottle gently for approximately 30 seconds and pouring off the rinse. The filter is then washed with 2x SSC + 0.1% SDS at room temperature for 20 to 30 minutes, with shaking.

The membrane is removed from the wash and placed on a dry piece of 3MM WHATMAN filter paper then wrapped in one layer of plastic wrap, placed on the autoradiography film, and exposed for about five hours depending upon a survey meter

indicating the level of radioactivity. The film is developed in an automatic film processor.

Control Hybridization with Normal Oligonucleotides

The purpose of this step is to ensure that the PCR products are transferred efficiently to the nylon membrane.

Following hybridization with the mutant oligonucleotide, as described in the Examples above, each nylon membrane is washed in 2X SSC, 0.1% SDS for 20 minutes at 65°C to melt off the mutant oligonucleotide probes. The nylon strips were then prehybridized together in 40 ml of TMAC hybridization solution for at least 1 hour at 52°C in a shaking water bath. 2-5 million counts of each of the normal labeled oligonucleotide probes plus 40 μl of 20μM stock of unlabeled normal oligonucleotide are added directly to the container containing the nylon membranes and the prehybridization solution. The filter and probes are hybridized at 52°C with shaking for at least 1 hour. Hybridization can be performed overnight, if necessary. The hybridization solution is poured off, and the nylon membrane is rinsed in 2X SSC, 0.1% SDS for 1 minute with gentle swirling by hand. The rinse is poured off and the membrane is washed in 2X SSC, 0.1% SDS at room temperature for 20 minutes with shaking.

The nylon membrane is removed and placed on a dry piece of 3MM WHATMAN filter paper. The nylon membrane is then wrapped in one layer of plastic wrap and placed on autoradiography film, and exposure is for at least 1 hour.

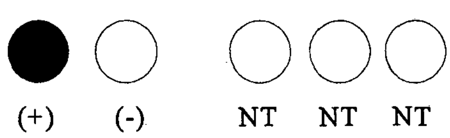
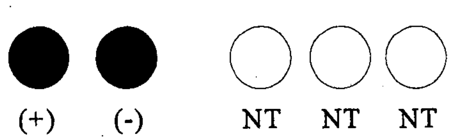
For each sample, adequate transfer to the membrane is indicated by a strong autoradiographic hybridization signal. For each sample, an absent or weak signal when hybridized with its normal oligonucleotide, indicates an unsuccessful transfer

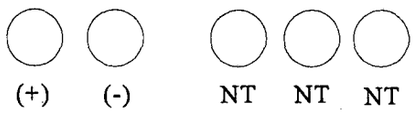
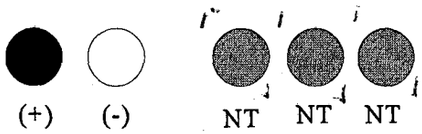
of PCR product, and it is a false negative. The ASO analysis must be repeated for any sample that did not successfully transfer to the nylon membrane.

Interpreting Results

After hybridizing with mutant oligonucleotides, the results for each exon are interpreted as follows:

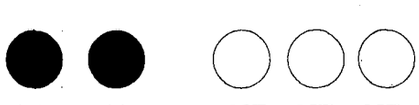
Table 4A

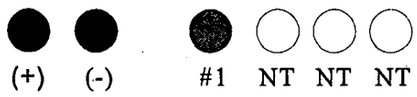
Result	Interpretation	Action
	All controls indicate assay is successful	Record results, dark circles are mutation positive, and all others are negative
	Assay not specific, mutant oligonucleotide hybridizing to normal DNA.	Rewash membrane 30 minutes longer at appropriate temp. and re-expose.

1		Mutant oligonucleotide probe is either washed off or did not label well enough, or PCR product is not transferred to membrane efficiently.	Rehybridize with remaining labeled oligonucleotide. If still no signal, perform normal oligonucleotide hyb. as per the Examples to test transfer of PCR to membrane.
		Positive and negative controls indicate assay is successful, but PCR is contaminated.	Perform standard clean up procedures for PCR contamination. Repeat assay.

After hybridization with normal oligonucleotides, interpret the results as follows:

Table 4B

6		Results indicate transfer of PCR products to membrane is successful.	Record results.

	Results indicate transfer of patient sample #1 is inefficient. May get false negative from this sample.	This sample will have to be transferred to another membrane and the assay repeated.

The sample #1 should be lighter than the controls. Patient samples containing a mutation are generally heterozygous and will hybridize to both the normal and mutant oligonucleotide probes. The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All references mentioned herein are incorporated by reference.

P190X

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: LESCALETT, JENNIFER L.
LAWRENCE, TAMMY
THURBER, DENISE B.
OLSEN, SHERI J.
ALLEN, ANTONETTE P.
WHITE, MARGA B.

(ii) TITLE OF INVENTION: CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2

(iii) NUMBER OF SEQUENCES: 24

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Oncormed, Inc.
(B) STREET: 205 Perry Parkway
(C) CITY: Gaithersburg
(D) STATE: MD
(E) COUNTRY: USA
(F) ZIP: 20877

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US

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(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: TARCZA, John E.

(B) REGISTRATION NUMBER: 33,638

(C) REFERENCE/DOCKET NUMBER: PA-0107

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 301-208-1888

(B) TELEFAX: 301-527-1539

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGGTACTTTA ATTTTGTCAC TT

22

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGCAGGCATG ACAGAGAA

18

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGAAGAACCA ACTTTGT

17

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGAAGAACGA ACTTTGT

17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTCAGATGTT ATTTTCCAAG C

21

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGTTAAATA ACCAGAAGCA C

21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCAAGCAATT TGAAGGT

17

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCAAGCAATG AAGGTAC

17

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCAAAGACCC TAAAGTACAG
20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CATCAAATAT TCCTTCTCTA AG

22

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACTTGTTACA CAAATCA

17

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACTTGTTAGA CAAATCA

17

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAAAATTCAG CCTTAGC

17

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATCAGAATGG TAGGAAT

17

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATTATTTGTA TGAAAAT

17

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ATTATTTGAA AATAATT

17

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TACAGCAAGT GGAAAGC

17

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAGTTTCAGT TTTACCAAT
19

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GAACTGAGCA TAGTCTT

17

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GAACTGAATA GTCTTCA

17

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ACTTTTTCTG ATGTTCCTGT G

21

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TAAAAATAGT GATTGGCAAC A

21

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CAGAAGCAGT AGAAATT

17

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CAGAAGCAGG TAGAAAT

17

We Claim:

1. An isolated oligonucleotide selected from the group consisting of:

a first oligonucleotide for detecting an amino acid change from proline to arginine at codon 655 of a BRCA2 gene sequence, wherein said first oligonucleotide specifically hybridizes to a region containing nucleotide number 2192 of the BRCA2 gene,

a second oligonucleotide for detecting a deletion of two nucleotides at nucleotide number 3772 of a BRCA2 gene sequence, wherein said second oligonucleotide specifically hybridizes to a region containing nucleotide number 3772 of the BRCA2 gene,

a third oligonucleotide for detecting an amino acid change from tryptophan to a stop codon at codon 1655 of a BRCA2 gene sequence, wherein said fourth oligonucleotide specifically hybridizes to a region containing nucleotide number 5193 of the BRCA2 gene,

a fourth oligonucleotide for detecting a deletion of four nucleotides at nucleotide number 5374 of a BRCA2 gene sequence, wherein said fifth oligonucleotide specifically hybridizes to a region containing nucleotide number 5374 of the BRCA2 gene,

a fifth oligonucleotide for detecting a deletion of two nucleotides at nucleotide number 6495 of a BRCA2 gene sequence, wherein said sixth oligonucleotide specifically hybridizes to a region containing nucleotide number 6495 of the BRCA2 gene, and

a sixth oligonucleotide for detecting an insertion of one nucleotide at nucleotide number 6909 of a BRCA2 gene sequence,

wherein said seventh oligonucleotide specifically hybridizes to a region containing nucleotide number 6909 of the BRCA2 gene.

12. ~~The~~ An isolated oligonucleotide ~~according to claim 1~~ wherein the oligonucleotide is capable of detecting a G at nucleotide number 2192 of a BRCA2 gene by specifically hybridizing to the region containing nucleotide number 2192 of the BRCA2 gene.

13. An isolated ~~wildtype allele specific~~ oligonucleotide ~~according to claim 2~~ having the sequence 5'TGA AGA ACC AAC TTT GT3', SEQ ID NO:3, or the complementary oligonucleotides thereto.

14. An isolated ~~mutant allele specific~~ oligonucleotide according to claim 12 having the sequence 5'TGA AGA ACG AAC TTT GT3', SEQ ID NO:4, or the complementary oligonucleotides thereto.

15. The isolated oligonucleotide ~~according to claim 1~~ wherein the oligonucleotide is capable of detecting ~~the~~ a deletion of TT at nucleotide number 3772 of a BRCA2 gene by specifically hybridizing to the region containing nucleotide number 3772 of the BRCA2 gene.

16. An isolated ~~wildtype allele specific.~~ oligonucleotide ~~according to claim 5~~ having the sequence 5'GCA AGC AAT TTG AAG GT3', SEQ ID NO:7, or the complementary oligonucleotides thereto.

17. An isolated ~~mutant allele specific~~ oligonucleotide according to claim 16 having the sequence 5'GCA AGC AAT GAA GGT AC3', SEQ ID NO:8, or the complementary oligonucleotides thereto.

7 8. ~~The~~ isolated oligonucleotide ~~according to claim 1~~ wherein the oligonucleotide is capable of detecting ~~the~~ substitution of G for C at nucleotide number 5193 by specifically hybridizing to the region containing nucleotide number 5193 of the BRCA2 gene.

8 9. An isolated ~~wildtype allele specific~~ oligonucleotide ~~according to claim 8~~ having the sequence 5'ACT TGT TAC ACA AAT CA3', SEQ ID NO:11, or complementary oligonucleotides thereto.

9 10. An isolated ~~mutant allele specific~~ oligonucleotide according to claim 8 having the sequence 5'ACT TGT TAG ACA AAT CA3', SEQ ID NO:12, or complementary oligonucleotides thereto.

11. The isolated oligonucleotide according to claim 1 wherein the oligonucleotide is capable of detecting a G at nucleotide number 5193 by specifically hybridizing to the region containing nucleotide number 5193 of the BRCA2 gene.

12. An isolated wildtype allele specific oligonucleotide according to claim 11 having the sequence 5'ACT TGT TAG ACA AAT CA3', SEQ ID NO:12, or complementary oligonucleotides thereto.

13. An isolated mutant allele specific oligonucleotide according to claim 11 having the sequence 5'ACT TGT TAC ACA AAT CA3', SEQ ID NO:11, or complementary oligonucleotides thereto.

10 14. ~~The~~ isolated oligonucleotide according to claim 1 wherein the oligonucleotide is capable of detecting ~~the~~

deletion of TATG at nucleotide number 5374 of a BRCA2 gene by specifically hybridizing to the region containing nucleotide number 5374 of the BRCA2 gene.

11 ~~15~~. An isolated ~~wildtype allele specific~~ oligonucleotide ~~according to claim 14~~ having the sequence 5'ATT ATT TGT ATG AAA AT3', SEQ ID NO:15, or the complementary oligonucleotides thereto.

12 ~~16~~. An isolated ~~mutant allele specific~~ oligonucleotide according to claim 10 having the sequence 5'ATT ATT TGA AAA TAA TT3', SEQ ID NO:16, or the complementary oligonucleotides thereto.

13 ~~17~~. ~~The~~ An isolated oligonucleotide ~~according to claim 1~~ wherein the oligonucleotide is capable of detecting ~~the~~ a deletion of GC at nucleotide number 6495 of a BRCA2 gene by specifically hybridizing to the region containing nucleotide number 6495 of the BRCA2 gene.

14 ~~18~~. An isolated ~~wildtype allele specific~~ oligonucleotide ~~according to claim 17~~ having the sequence 5'GAA CTG AGC ATA GTC TT3', SEQ ID NO:19, or the complementary oligonucleotides thereto.

15 ~~19~~. An isolated ~~mutant allele specific~~ oligonucleotide according to claim 13 having the sequence 5'GAA CTG AAT AGT CTT CA3', SEQ ID NO:20, or the complementary oligonucleotides thereto.

16 ~~20~~. ~~The~~ An isolated oligonucleotide ~~according to claim 1~~ wherein the oligonucleotide is capable of detecting ~~the~~ an insertion of G at nucleotide number 6909 of a BRCA2 gene by specifically

hybridizing to the region containing nucleotide number 6909 of the BRCA2 gene.

17 ~~21~~. An isolated ~~wildtype allele specific~~ oligonucleotide ~~according to claim 20~~ having the sequence 5'CAG AAG CAG TAG AAA TT3', SEQ ID NO:23, or the complementary oligonucleotides thereto.

18 ~~22~~. An isolated ~~mutant allele specific~~ oligonucleotide according to claim 16 having the sequence 5'CAG AAG CAG GTA GAA AT3', SEQ ID NO:24, or the complementary oligonucleotides thereto.

19 ~~23~~. The isolated oligonucleotide according to ~~claim 1~~ any one of claims 1-3, 5-8, 10, 13, 14, 17 and 20 further comprising a label bound thereto.

20 ~~24~~. The isolated oligonucleotide according to claim 19 wherein the label is selected from the group consisting of a radiolabel, a fluorescent label, a bioluminescent label, a chemiluminescent label, an enzyme label and a ligand label.

25. An isolated oligonucleotide primer capable of hybridizing to a BRCA2 gene and initiating DNA synthesis to extend the primer, said isolated oligonucleotide primer comprising the sequence ~~hybridizing~~ to the BRCA2 gene at a region overlapping the region where one of the following oligonucleotides hybridizes:

BRCA-2-11F: 5'TGG TAC TTT AAT TTT GTC ACT T3' (SEQ ID NO:1),

BRCA-2-11R: 5'TGC AGG CAT GAC AGA GAA T3' (SEQ ID NO:2),

BRCA-2-11F: 5'CTC AGA TGT TAT TTT CCA AGC3' (SEQ ID NO:5),

BRCA-2-11R: 5'CTG TTA AAT AAC CAG AAG CAC3' (SEQ ID NO:6),

BRCA-2-11F: 5'GCA AAG ACC CTA AAG TAC AG3' (SEQ ID NO:9),

BRCA-2-11R: 5'CAT CAA ATA TTC CTT CTC TAA G3' (SEQ ID NO:10),

BRCA-2-11F: 5'GAA AAT TCA GCC TTA GC3' (SEQ ID NO:13),
BRCA-2-11R: 5'ATC AGA ATG GTA GGA AT3' (SEQ ID NO:14),
BRCA-2-11F: 5'TAC AGC AAG TGG AAA GC3' (SEQ ID NO:17),
BRCA-2-11R: 5'AAG TTT CAG TTT TAC CAA T3' (SEQ ID NO:18),
BRCA-2-11F: 5'ACT TTT TCT GAT GTT CCT GTG3' (SEQ ID NO:21), or
BRCA-2-11R: 5'TAA AAA TAG TGA TTG GCA ACA3' (SEQ ID NO:22).

26. An isolated oligonucleotide primer according to claim 25 which is forward primer comprising the sequence BRCA-2-11F: 5'TGG TAC TTT AAT TTT GTC ACT T3' (SEQ ID NO:1) ~~or a~~ substantially similar sequence capable of hybridizing to ~~a~~ complementary polynucleotide and initiating DNA synthesis along the complementary polynucleotide.

27. An isolated oligonucleotide primer according to claim 25 which is reverse primer comprising the sequence BRCA-2-11R: 5'TGC AGG CAT GAC AGA GAA T3' (SEQ ID NO:2) ~~or a substantially similar sequence capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis along the complementary polynucleotide.~~

28. An isolated oligonucleotide primer according to claim 25 which is forward primer comprising the sequence BRCA-2-11F: 5'CTC AGA TGT TAT TTT CCA AGC3' (SEQ ID NO:5) or a substantially similar sequence capable of hybridizing to ~~a~~ complementary polynucleotide and initiating DNA synthesis along the complementary polynucleotide.

29. An isolated oligonucleotide primer according to claim 25 which is reverse primer comprising the sequence BRCA-2-11R: 5'CTG TTA AAT AAC CAG AAG CAC3' (SEQ ID NO:6) ~~or a~~

B substantially similar sequence capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis along the complementary polynucleotide.

Rule 126 30 32. An isolated oligonucleotide primer according to claim 25 which is forward primer comprising the sequence BRCA-2-11F: 5'GCA AAG ACC CTA AAG TAC AG3', SEQ ID NO:9, or a B substantially similar sequence capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis along the complementary polynucleotide.

Rule 126 31 33. An isolated oligonucleotide primer according to claim 25 which is reverse primer comprising the sequence BRCA-2-11R: 5'CAT CAA ATA TTC CTT CTC TAA G3', SEQ ID NO:10, or a B substantially similar sequence capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis along the complementary polynucleotide.

Rule 126 32 34. An isolated oligonucleotide primer according to claim 25 which is forward primer comprising the sequence BRCA-2-11F: 5'GAA AAT TCA GCC TTA GC3' (SEQ ID NO:13) or a B substantially similar sequence capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis along the complementary polynucleotide.

Rule 126 39 35. An isolated oligonucleotide primer according to claim 25 which is reverse primer comprising the sequence BRCA-2-11R: 5'ATC AGA ATG GTA GGA AT3' (SEQ ID NO:14) or a B substantially similar sequence capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis along the complementary polynucleotide.

Rule 126 34 36. An isolated oligonucleotide primer according to claim 25 which is forward primer comprising the sequence BRCA-2-11F: 5'TAC AGC AAG TGG AAA GC3' (SEQ ID NO:17) or a B substantially similar sequence capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis along the complementary polynucleotide.

Rule 126 35 37. An isolated oligonucleotide primer according to claim 25 which is reverse primer comprising the sequence BRCA-2-11R: 5'AAG TTT CAG TTT TAC CAA T3' (SEQ ID NO:18) or a B substantially similar sequence capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis along the complementary polynucleotide.

Rule 126 36 38. An isolated oligonucleotide primer according to claim 25 which is forward primer comprising the sequence BRCA-2-11F: 5'ACT TTT TCT GAT GTT CCT GTG3' (SEQ ID NO:21) or a B substantially similar sequence capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis along the complementary polynucleotide.

Rule 126 37 39. An isolated oligonucleotide primer according to claim 25 which is reverse primer comprising the sequence BRCA-2-11R: 5'TAA AAA TAG TGA TTG GCA ACA3' (SEQ ID NO:22) or a B substantially similar sequence capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis along the complementary polynucleotide.

Rule 126 38 40. A pair of isolated oligonucleotide primers which specifically hybridize to the BRCA2 gene, one of which can ~~effectively~~ specifically hybridize to exon 11 of the BRCA2 gene, and the

other can effectively hybridize to either exon 11 or one of the two intron regions flanking exon 11.

41. The pair of primers according to claim 40 wherein said pair comprises a primer having the sequence BRCA-2-11F: 5'TGG TAC TTT AAT TTT GTC ACT T3' (SEQ ID NO:1) and a primer comprising the sequence BRCA-2-11R: 5'TGC AGG CAT GAC AGA GAA T3' (SEQ ID NO:2), or substantially similar sequences capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis.

42. The pair of primers according to claim 40 wherein said pair comprises a primer having the sequence BRCA-2-11F: 5'CTC AGA TGT TAT TTT CCA AGC3' (SEQ ID NO:5) and a primer comprising the sequence BRCA-2-11R: 5'CTG TTA AAT AAC CAG AAG CAC3' (SEQ ID NO:6), or substantially similar sequences capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis.

44. The pair of primers according to claim 40 wherein said pair comprises a primer having the sequence BRCA-2-11F: 5'GCA AAG ACC CTA AAG TAC AG3', SEQ ID NO:9, and a primer comprising the sequence BRCA-2-11R:5'CAT CAA ATA TTC CTT CTC TAA G3', SEQ ID NO:10, or substantially similar sequences capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis.

45. The pair of primers according to claim 40 wherein said pair comprises a primer having the sequence BRCA-2-11F: 5'GAA AAT TCA GCC TTA GC3' (SEQ ID NO:13) and a primer comprising the sequence BRCA-2-11R: 5'ATC AGA ATG GTA GGA AT3'

(SEQ ID NO:14), or substantially similar sequences capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis.

46. The pair of primers according to claim 40 wherein said pair comprises a primer having the sequence BRCA-2-11F: 5'TAC AGC AAG TGG AAA GC3' (SEQ ID NO:17) and a primer comprising the sequence BRCA-2-11R: 5'AAG TTT CAG TTT TAC CAA T3' (SEQ ID NO:18), or substantially similar sequences capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis.

47. The pair of primers according to claim 40 wherein said pair comprises a primer having the sequence BRCA-2-11F: 5'ACT TTT TCT GAT GTT CCT GTG3' (SEQ ID NO:21) and a primer comprising the sequence BRCA-2-11R: 5'TAA AAA TAG TGA TTG GCA ACA3' (SEQ ID NO:22), or substantially similar sequences capable of hybridizing to a complementary polynucleotide and initiating DNA synthesis.

48. The pair of isolated oligonucleotide primers according to claim 40, wherein each primer is bound to a label.

49. The pair of primers according to claim 48 wherein each of said label is selected from the group consisting of a radiolabel, a fluorescent label, a bioluminescent label a chemiluminescent label, an enzyme label and a ligand label.

50. The isolated oligonucleotide primer according to claim 25 bound to a label.

51. The primer according to claim ~~48~~ 47 wherein said label is selected from the group consisting of a radiolabel, a fluorescent label a bioluminescent label a chemiluminescent label, an enzyme label and a ligand label.

52. A method for determining the presence or absence of a sequence variation in ~~a gene sample,~~ the BRCA2 gene at nucleotide number 2192, 3772, 5193, 5374, 6495 or 6909 comprising:

(a) performing an allele specific detection assay for the presence or absence of one or more ~~predetermined~~ said sequence variations; and

(b) determining the presence or absence of a sequence variation in the BRCA2 gene sample at nucleotide number 2192, 3772, 5193, 5374, 6495 or 6909.

53. The method according to claim ~~52~~ 24 wherein the ~~predetermined~~ said sequence variation is C2192G, 3772delTT, C5193G, 5374del4, 6495delGC or 6909insG.

54. The method of Claim ~~52~~ 24 wherein the allele specific detection assay is performed as part of a multiplex amplification assay format.

55. The method of Claim ~~52~~ 24 wherein the allele specific ~~sequence-based~~ detection assay is performed using a dot blot format, reverse dot blot format, a MASDA format, or a chip array format.

56. The method according to Claim ~~52~~ 24 further comprising

(a) performing an allele specific detection assay for the presence or absence of one or more reference sequences without ~~the predetermined~~ said sequence variations.

57. The method according to claim 56 wherein said reference sequence is a BRCA2 coding sequence.

58. The method according to Claim 56 wherein said reference sequence is a BRCA2 genomic sequence.

59. The method according to Claim 56 wherein said reference sequence is one or more exons of the BRCA2 gene.

60. A chip array having "n" cells for performing allele specific sequence-based techniques comprising;

a solid phase chip with "n" cells and

oligonucleotides having "n" different nucleotide sequences, at least one sequence being capable of specifically hybridizing to the oligonucleotide of claim 1,

wherein "n" is an interger greater than one,

wherein said oligonucleotides are bound to said solid phase chip in a manner which permits said oligonucleotides to effectively hybridize to complementary oligonucleotides or polynucleotides, and

wherein oligonucleotides having different nucleotide sequence are bound to said solid phase chip at different cells so that a particular location on said solid phase chip contains oligonucleotides having one nucleotide sequence exclusively.

61. A method of detecting a predisposition or higher susceptibility to cancer in an individual, comprising:

(a) digesting DNA from said individual into fragments;

(b) separating said DNA fragments obtained from said digestion;

(c) detecting a DNA fragment containing nucleotide number 2192, 3772, 5193, 5374, 6495 or 6909 of the BRCA2 gene sequence or a sequence variation at nucleotide number 2192, 3772, 5193, 5374, 6495 or 6909 of the BRCA2 gene sequence by sequencing;

(d) comparing the ~~DNA~~ sequence of said fragment ~~sequence~~ with the BRCA2 gene sequence ~~for~~ to determine the presence or absence of a sequence variation at nucleotide number 2192, 3772, 5193, 5374, 6495 or 6909, wherein the presence of a sequence variation indicates a predisposition or higher susceptibility to cancer.

~~62~~. A method according to claim ~~61~~ further comprising amplifying said DNA fragments prior to the detecting step (c).

~~63~~. A method according to claim ~~61~~ wherein the sequence variation is amplified with an oligonucleotide primer having a sequence of:

5'TGG TAC TTT AAT TTT GTC ACT T3' SEQ ID NO:1,

5'TGC AGG CAT GAC AGA GAA T3' SEQ ID NO:2,

5'CTC AGA TGT TAT TTT CCA AGC3' SEQ ID NO:5,

5'CTG TTA AAT AAC CAG AAG CAC3' SEQ ID NO:6,

5'GCA AAG ACC CTA AAG TAC AG3' SEQ ID NO:9,

5'CAT CAA ATA TTC CTT CTC TAA G3' SEQ ID NO:10,

5'GAA AAT TCA GCC TTA GC3' SEQ ID NO:13,

5'ATC AGA ATG GTA GGA AT3' SEQ ID NO:14,

5'TAC AGC AAG TGG AAA GC3' SEQ ID NO:17,

5'AAG TTT CAG TTT TAC CAA T3' SEQ ID NO:18,

5'ACT TTT TCT GAT GTT CCT GTG3' SEQ ID NO:21,

5'TAA AAA TAG TGA TTG GCA ACA3' SEQ ID NO:22 or

a sequence capable of specific hybridization to and initiation of DNA synthesis on a complementary oligonucleotide or polynucleotide.

64. A method according to claim 63 wherein said oligonucleotide primer is labeled with a radiolabel, a fluorescent label a bioluminescent label, a chemiluminescent label, an enzyme label, or a ligand label.

65. A method of detecting a predisposition or higher susceptibility to cancer in an individual, comprising;

(a) digesting DNA from said individual,

(b) separating DNA fragments obtained from said digestion,

(c) hybridizing a DNA fragment with an allele specific oligonucleotide having a nucleotide sequence capable of hybridizing to either a polynucleotide having a sequence contained within the BRCA2 gene sequence or a sequence variation at nucleotide number 2192, 3772, 5193, 5374, 6495 or 6909 of the BRCA2 gene sequence, and

(d) correlating the presence or absence of said sequence variation with the respective presence or absence of the BRCA2 gene, thereby determining a predisposition or higher susceptibility to cancer.

66. A method according to claim 65 herein said allele specific oligonucleotide is:

5'TGA AGA ACC AAC TTT GT3' SEQ ID NO:3,

5'TGA AGA ACG AAC TTT GT3' SEQ ID NO:4,

5'GCA AGC AAT TTG AAG GT3' SEQ ID NO:7,

5'GCA AGC AAT GAA GGT AC3' SEQ ID NO:8,

5'ACT TGT TAC ACA AAT CA3' SEQ ID NO:11,

5'ACT TGT TAG ACA AAT CA3' SEQ ID NO:12,

5'ATT ATT TGT ATG AAA AT3' SEQ ID NO:15,

5'ATT ATT TGA AAA TAA TT3' SEQ ID NO:16,

5'GAA CTG AGC ATA GTC TT3' SEQ ID NO:19,

5'GAA CTG AAT AGT CTT CA3' SEQ ID NO:20,

5'CAG AAG CAG TAG AAA TT3' SEQ ID NO:23,

5'CAG AAG CAG GTA GAA AT3' SEQ ID NO:24, or

a sequence capable of specific hybridization to a region on the BRCA2 gene or said sequence variation of the BRCA2 gene overlapping the region complementary to SEQ ID NO:3, 4, 7, 8, 11, 12, 15, 16, 19, 20, 23, or 24.

67. A method according to claim ~~65~~ further comprising amplifying said DNA fragment prior to sequencing.

68. A method according to claim ~~65~~ wherein said oligonucleotide is labeled with a radiolabel, a fluorescent label, a bioluminescent label, a chemiluminescent label, an enzyme label, or a ligand label.

69. A kit comprising a carrier means being compartmentalized to receive in close confinement one or more container means, and at least one container means,

wherein said at least one container means contains the oligonucleotide of ~~claim 1~~.

70. The kit acording to claim 69 further comprising at least one container means containing the oligonucleotide primer of claim 25.

72. The kit acording to claim 69 further comprising at least one container means containing the pair of oligonucleotide primers of claim 40.

73. A kit comprising a carrier means being compartmentalized to receive in close confinement one or more container means, and at least one container means,

wherein at least one container means contains the oligonucleotide primer of claim 25.

74. A kit comprising a carrier means being compartmentalized to receive in close confinement one or more container means, and at least one container means,

wherein at least one container means contains the pair of oligonucleotide primers of claim 40.

75. An isolated DNA sequence consisting essentially of DNA coding for at least a part of a BRCA2 gene containing at least one mutation from the list: C2192G, 3772delTT, C5193G, 5374del4, 6495delGC or 6909insG, or an isolated DNA sequence complementary thereto.

76. A vector comprising the isolated DNA sequence according to claim 75 linked to a vector by at least one of the termini of the isolated DNA sequence.

77. An isolated DNA sequence according to claim 75, wherein the isolated DNA sequence contains a sequence complementary to at least one of the following: SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO12:, SEQ ID NO:16, SEQ ID NO:20, or SEQ ID NO:24, or an isolated DNA sequence complementary thereto.

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78. A vector comprising the isolated DNA sequence a according to claim 73 ~~77~~ linked to a vector by at least one of the termini of the isolated DNA sequence.

C2192G, 3772delTT, C5193G, 5374 del4, 6495del GC, or 6909 insG

44 ~~75~~ 79. A method of determining whether a mutation is present in a BRCA2 gene comprising sequencing at least a portion of the BRCA2 gene containing either:

a sequence complementary to SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:12, SEQ ID NO16:, SEQ ID NO:20 or SEQ ID NO:24, or an isolated DNA sequence ~~complementary thereto~~ which is the complement strand, or

at least one mutation from the list: C2192G, 3772delTT, C5193G, 5374del4, 6495delGC or 6909insG.

?

COMBINED DECLARATION AND POWER OF ATTORNEY

(Original, Design, National Stage of PCT or CIP Application)

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2

the specification of which:

Regular or Design Application

(a) [X] is attached hereto.
(b) [ ] was filed on as Application Serial No. and was amended on .

PCT Filed Application Entering National Stage

(c) [ ] was described and claimed in International Application No. filed on and as amended on .

Acknowledgment of Review of Papers and Duty of Candor

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of the subject matter claimed in this application in accordance with Title 37, Code of Federal Regulations § 1.56.

[ ] In compliance with this duty there is attached an information disclosure statement. 37 CFR 1.97.

Priority Claim

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed

(d) [X] no such applications have been filed.
(e) [ ] such applications have been filed as follows:

BA R & BOTTS, L.L.P.
E NO.

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION				
COUNTRY	APPLICATION NO.	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)	PRIORITY CLAIMED UNDER 37 USC 119
				[ ] YES NO [ ]
				[ ] YES NO [ ]
				[ ] YES NO [ ]
ALL FOREIGN APPLICATION[S], IF ANY, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO SAID APPLICATION				
				[ ] YES NO [ ]
				[ ] YES NO [ ]
				[ ] YES NO [ ]

Claim for Benefit of Prior U.S. Provisional Application(s)

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

Provisional Application Number	Filing Date
60/059,595	September 23, 1997

Continuation-In-Part

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information as defined in Title 37, Code of Federal Regulations, § 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.) (Filing Date) (Status) (patented, pending, abandoned)

(Application Serial No.) (Filing Date) (Status) (patented, pending, abandoned)

Power of Attorney

As a named inventor, I hereby appoint Dana M. Raymond, Reg. No. 18,540; Frederick C. Carver, Reg. No. 17,021; Francis J. Hone, Reg. No. 18,662; Joseph D. Garon, Reg. No. 20,420; Arthur S. Tenser, Reg. No. 18,839; Ronald B. Hildreth, Reg. No. 19,498; Thomas R. Nesbitt, Jr., Reg. No. 22,075; Robert Neuner, Reg. No. 24,316; Richard G. Berkley, Reg. No. 25,465; Richard S. Clark, Reg. No. 26,154; Thomas D. MacBlain, Reg. No. 24,583; Bradley B. Geist, Reg. No. 27,551; James J. Maune, Reg. No. 26,946; John D. Murnane, Reg. No. 29,836, Henry Tang, Reg. No. 29,705, Robert C. Scheinfeld, Reg. No. 31,300, John A. Fogarty, Jr., Reg. No. 22,348, Louis S. Sorell, Reg. No. 32,439 and Rochelle K. Seide, Reg. No. 32,300 of the firm of BAKER & BOTTS, L.L.P., with offices at 30 Rockefeller Plaza, New York, New York 10112 and R. Thomas Gallegos, Reg. No. 32,692 and John E. Tarcza, Reg. No. 33,638 of OncorMed, Inc. with offices at 205 Perry Parkway, Gaithersburg, Maryland 20877, as attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith

BAKER & BOTTS, L.L.P.
FILE NO.

SEND CORRESPONDENCE TO:	DIRECT TELEPHONE CALLS TO:
BAKER & BOTTS, L.L.P. 30 ROCKEFELLER PLAZA, NEW YORK, N.Y. 10112	BAKER & BOTTS, L.L.P. (212) 705-5000

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

FULL NAME OF SOLE OR FIRST INVENTOR	LAST NAME Lescallett	FIRST NAME Jennifer	MIDDLE NAME Lee	
RESIDENCE & CITIZENSHIP	CITY Great Falls	STATE or FOREIGN COUNTRY Virginia	COUNTRY OF CITIZENSHIP USA	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 10613 Good Spring Ave.	CITY Great Falls	STATE or COUNTRY VA	ZIP CODE 22066
DATE 11/25/97	SIGNATURE OF INVENTOR			
FULL NAME OF SECOND JOINT INVENTOR, IF ANY	LAST NAME Lawrence	FIRST NAME Tammy	MIDDLE NAME N/A	
RESIDENCE & CITIZENSHIP	CITY Laurel	STATE or FOREIGN COUNTRY Maryland	COUNTRY OF CITIZENSHIP USA	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 186 Jill Lane #101	CITY Laurel	STATE or COUNTRY MD	ZIP CODE 20724
DATE 11/25/97	SIGNATURE OF INVENTOR			
FULL NAME OF THIRD JOINT INVENTOR, IF ANY	LAST NAME Allen	FIRST NAME Antonette	MIDDLE NAME Preisinger	
RESIDENCE & CITIZENSHIP	CITY Severn	STATE or FOREIGN COUNTRY Maryland	COUNTRY OF CITIZENSHIP USA	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 1405 Hawaii Avenue	CITY Severn	STATE or COUNTRY MD	ZIP CODE 21144
DATE 11/25/97	SIGNATURE OF INVENTOR			
FULL NAME OF FOURTH JOINT INVENTOR, IF ANY	LAST NAME Olson	FIRST NAME Sheri	MIDDLE NAME Jon	
RESIDENCE & CITIZENSHIP	CITY Falls Church	STATE or FOREIGN COUNTRY Virginia	COUNTRY OF CITIZENSHIP USA	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 2854 Yarling Court	CITY Falls Church	STATE or COUNTRY VA	ZIP CODE 22024
DATE 11/25/97	SIGNATURE OF INVENTOR			

BAK[illegible]R & BOTTS, [illegible].P.
F[illegible] NO.

FULL NAME OF FIFTH JOINT INVENTOR, IF ANY	LAST NAME Thurber	FIRST NAME Denise	MIDDLE NAME Bernadette	
RESIDENCE & CITIZENSHIP	CITY Silver Spring	STATE or FOREIGN COUNTRY Maryland	COUNTRY OF CITIZENSHIP USA	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 2813 Parker Court	CITY Silver Spring	STATE or COUNTRY MD	ZIP CODE 20902
DATE 11/25/97	SIGNATURE OF INVENTOR Denise Bernadette Thurber			
FULL NAME OF SIXTH JOINT INVENTOR, IF ANY	LAST NAME White	FIRST NAME Marga	MIDDLE NAME Belle	
RESIDENCE & CITIZENSHIP	CITY Frederick	STATE or FOREIGN COUNTRY Maryland	COUNTRY OF CITIZENSHIP USA	
POST OFFICE ADDRESS	POST OFFICE ADDRESS 8323 Sharon Drive	CITY Frederick	STATE or COUNTRY MD	ZIP CODE 21701
DATE 11/26/97	SIGNATURE OF INVENTOR Marga Belle White			
FULL NAME OF SEVENTH JOINT INVENTOR, IF ANY	LAST NAME	FIRST NAME	MIDDLE NAME	
RESIDENCE & CITIZENSHIP	CITY	STATE or FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP	
POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE or COUNTRY	ZIP CODE
DATE	SIGNATURE OF INVENTOR			

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27 (c)) - SMALL BUSINESS CONCERN			Docket No.
Serial No.	Filing Date	Patent No.	Issue Date

Applicant/ Patentee: Jennifer L. Lescallet, Antonette C.P. Allen, Tammy Lawrence, Sheri J. Olson, Denise B. Thurber, and Marga B. White

Invention:

CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2

I hereby declare that I am:

- ☐ the owner of the small business concern identified below:
- ☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: Oncormed, Inc.
ADDRESS OF CONCERN: 205 Perry Parkway, Gaithersburg, MD 20877

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the above identified invention described in:

- ☒ the specification filed herewith with title as listed above.
- ☐ the application identified above.
- ☐ the patent identified above.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed on the next page and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

☒ no such person, concern or organization exists.
☐ each such person, concern or organization is listed below.

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Doug Dolginow, M.D.

TITLE OF PERSON SIGNING OTHER THAN OWNER: President & Chief Operating Officer

ADDRESS OF PERSON SIGNING:
c/o Oncormed, Inc.
205 Perry Parkway
Gaithersburg, MD 20877

SIGNATURE: D. Dolginow DATE: 25 November 1997

RAW SEQUENCE LISTING
PATENT APPLICATION *US/08/984,034*

OIPE
#2
DATE: 01/17/98
TIME: 16:39:31

INPUT SET: S22691.raw

This Raw Listing contains the General Information Section and up to the first 5 pages.

ENTERED

SEQUENCE LISTING

(1) General Information:

(i) APPLICANT: LESCALETT, JENNIFER L.
LAWRENCE, TAMMY
THURBER, DENISE B.
OLSEN, SHERI J.
ALLEN, ANTONETTE P.
WHITE, MARGA B.

(ii) TITLE OF INVENTION: CANCER SUSCEPTIBILITY MUTATIONS OF BRCA1

(iii) NUMBER OF SEQUENCES: 24

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Oncormed, Inc.
(B) STREET: 205 Perry Parkway
(C) CITY: Gaithersburg
(D) STATE: MD
(E) COUNTRY: USA
(F) ZIP: 20877

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: TARCZA, John E.
(B) REGISTRATION NUMBER: 33,638
(C) REFERENCE/DOCKET NUMBER: PA-0107

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 301-208-1888
(B) TELEFAX: 301-527-1539

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGGTACTTTA ATTTTGTCAC TT 22

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGCAGGCATG ACAGAGAA 18

(2) INFORMATION FOR SEQ ID NO:3:

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100
101       (i) SEQUENCE CHARACTERISTICS:
102            (A) LENGTH: 17 base pairs
103            (B) TYPE: nucleic acid
104            (C) STRANDEDNESS: single
105            (D) TOPOLOGY: linear
106
107      (ii) MOLECULE TYPE: other nucleic acid
108            (A) DESCRIPTION: /desc = "PROBE"
109
110     (iii) HYPOTHETICAL: NO
111
112      (iv) ANTI-SENSE: YES
113
114       (v) FRAGMENT TYPE: internal
115
116      (vi) ORIGINAL SOURCE:
117            (A) ORGANISM: HOMO SAPIENS
118            (B) STRAIN: BRCA2
119
120
121
122     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
123
124  TGAAGAACCA ACTTTGT                                                  17
125
126  (2) INFORMATION FOR SEQ ID NO:4:
127
128       (i) SEQUENCE CHARACTERISTICS:
129            (A) LENGTH: 17 base pairs
130            (B) TYPE: nucleic acid
131            (C) STRANDEDNESS: single
132            (D) TOPOLOGY: linear
133
134      (ii) MOLECULE TYPE: other nucleic acid
135            (A) DESCRIPTION: /desc = "PROBE"
136
137     (iii) HYPOTHETICAL: NO
138
139      (iv) ANTI-SENSE: NO
140
141       (v) FRAGMENT TYPE: internal
142
143      (vi) ORIGINAL SOURCE:
144            (A) ORGANISM: HOMO SAPIENS
145            (B) STRAIN: BRCA2
146
147
148
149     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
150
151  TGAAGAACGA ACTTTGT                                                  17
152
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153  (2) INFORMATION FOR SEQ ID NO:5:
154
155       (i) SEQUENCE CHARACTERISTICS:
156            (A) LENGTH: 21 base pairs
157            (B) TYPE: nucleic acid
158            (C) STRANDEDNESS: single
159            (D) TOPOLOGY: linear
160
161      (ii) MOLECULE TYPE: other nucleic acid
162            (A) DESCRIPTION: /desc = "PRIMER"
163
164     (iii) HYPOTHETICAL: NO
165
166      (iv) ANTI-SENSE: NO
167
168       (v) FRAGMENT TYPE: internal
169
170      (vi) ORIGINAL SOURCE:
171            (A) ORGANISM: HOMO SAPIENS
172            (B) STRAIN: BRCA2
173
174
175
176      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
177
178  CTCAGATGTT ATTTTCCAAG C                                              21
179
180  (2) INFORMATION FOR SEQ ID NO:6:
181
182       (i) SEQUENCE CHARACTERISTICS:
183            (A) LENGTH: 21 base pairs
184            (B) TYPE: nucleic acid
185            (C) STRANDEDNESS: single
186            (D) TOPOLOGY: linear
187
188      (ii) MOLECULE TYPE: other nucleic acid
189            (A) DESCRIPTION: /desc = "PRIMER"
190
191     (iii) HYPOTHETICAL: NO
192
193      (iv) ANTI-SENSE: NO
194
195       (v) FRAGMENT TYPE: internal
196
197      (vi) ORIGINAL SOURCE:
198            (A) ORGANISM: HOMO SAPIENS
199            (B) STRAIN: BRCA2
200
201
202
203      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
204
205  CTGTTAAATA ACCAGAAGCA C                                              21
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206
207  (2) INFORMATION FOR SEQ ID NO:7:
208
209       (i) SEQUENCE CHARACTERISTICS:
210            (A) LENGTH: 17 base pairs
211            (B) TYPE: nucleic acid
212            (C) STRANDEDNESS: single
213            (D) TOPOLOGY: linear
214
215      (ii) MOLECULE TYPE: other nucleic acid
216            (A) DESCRIPTION: /desc = "PROBE"
217
218     (iii) HYPOTHETICAL: NO
219
220      (iv) ANTI-SENSE: NO
221
222       (v) FRAGMENT TYPE: internal
223
224      (vi) ORIGINAL SOURCE:
225            (A) ORGANISM: HOMO SAPIENS
226            (B) STRAIN: BRCA2
227
228
229
230      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
231
232  GCAAGCAATT TGAAGGT                                              17
233
234  (2) INFORMATION FOR SEQ ID NO:8:
235
236       (i) SEQUENCE CHARACTERISTICS:
237            (A) LENGTH: 17 base pairs
238            (B) TYPE: nucleic acid
239            (C) STRANDEDNESS: single
240            (D) TOPOLOGY: linear
241
242      (ii) MOLECULE TYPE: other nucleic acid
243            (A) DESCRIPTION: /desc = "PROBE"
244
245     (iii) HYPOTHETICAL: NO
246
247      (iv) ANTI-SENSE: NO
248
249       (v) FRAGMENT TYPE: internal
250
251      (vi) ORIGINAL SOURCE:
252            (A) ORGANISM: HOMO SAPIENS
253            (B) STRAIN: BRCA2
254
255
256
257      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
258
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SEQUENCE VERIFICATION REPORT
PATENT APPLICATION ***US/08/984,034***

DATE: 01/17/98
TIME: 16:39:58

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Line	Error	Original Text
31	Wrong application Serial Number	(A) APPLICATION NUMBER: US

Pre Amdt
3/9.
Rnda

FILE NO. A31421-2880/2

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Jennifer L. Lescallet, et al.	
Serial No.	:	08/984,034	Examiner:
Filed	:	December 2, 1997	Group Art Unit:
For	:	CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2	

PRELIMINARY AMENDMENT

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

February 17, 1998
Date of Deposit

Janet M. MacLeod
Agent Name

35,263
PTO Registration No.

Janet M. MacLeod
Signature

February 17, 1998
Date of Signature

Assistant Commissioner for Patents

Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application, please amend the claims as follows:

IN THE CLAIMS:

Please renumber Claims 32-42 as 30-40, respectively.

Please renumber Claims 44-70 as 41-67, respectively.

Please renumber Claims 72-79 as 68-75, respectively.

Claim 24, line 3, after "fluorescent label" insert --,--.

Claim 41, line 1, delete "40" and substitute therefor --38--.

Claim 42, line 1, delete "40" and substitute therefor --38--.

Claim 44, line 1, delete "40" and substitute therefor --38--.

Claim 45, line 1, delete "40" and substitute therefor --38--.

Claim 46, line 1, delete "40" and substitute therefor --38--.

Claim 47, line 1, delete "40" and substitute therefor --38--.

Claim 48, line 2, delete "40" and substitute therefor --38--.

Claim 49, line 1, delete "48" and substitute therefor --45--.

line 3, after "fluorescent label" insert --,--.

Claim 51, line 1, delete "48" and substitute therefor --45--.

Claim 53, line 1, delete "52" and substitute therefor --49--.

Claim 54, line 1, delete "52" and substitute therefor --49--.

Claim 55, line 1, delete "52" and substitute therefor --49--.

Claim 56, line 1, delete "52" and substitute therefor --49--.

Claim 57, line 1, delete "56" and substitute therefor --53--.

Claim 58, line 1 delete "56" and substitute therefor --53--.

Claim 59, line 1, delete "56" and substitute therefor --53--.

Claim 60, line 7, delete "interger" and substitute therefor --integer--.

line 13, delete "sequence" and substitute therefor --sequences--.

Claim 62, line 1, delete "61" and substitute therefor --58--.

Claim 63, line 1, delete "61" and substitute therefor --58--.

Claim 64, line 1, delete "63" and substitute therefor --60--.

Claim 66, line 1, delete "65 herein" and substitute therefor --62 wherein--.

Claim 67, line 1, delete "65" and substitute therefor --62--.

Claim 68, line 1, delete "65" and substitute therefor --62--.

line 3, after the first occurrence of "label" insert --,--.

Claim 74, line 5, delete "40" and substitute therefor --38--.

Claim 76, line 2, delete "75" and substitute therefor --71--.

Claim 77, line 1, delete "75" and substitute therefor --71--.

Claim 78, line 2, delete "77" and substitute therefor --73--.

PATENT

70. (Amended) The kit [acording] according to claim [69] 66 further comprising at least one container means containing [the oligonucleotide primer of claim 25]

an isolated oligonucleotide primer capable of hybridizing to a BRCA2 and initiating DNA synthesis to extend the primer, said isolated oligonucleotide primer hybridizing to the BRCA2 gene at a region overlapping the region where one of the following oligonucleotides hybridizes:

BRCA-2-11F: 5'TGG TAC TTT AAT TTT GTC ACT T3' (SEQ ID NO:1),

BRCA-2 11R: 5'TGC AGG CAT GAC AGA GAA T3' (SEQ ID NO: 2),

BRCA-2-11F: 5'CTC AGA TGT TAT TTT CAA AGC3' (SEQ ID NO:5),

BRCA-2-11R: 5'CTG TTA AAT AAC CAG AAG CAC3' (SEQ ID NO: 6),

BRCA-2-11F: 5'GCA AAG ACC CTA AAG TAC AG3' (SEQ ID NO:9),

BRCA-2-11R: 5'CAT CAA ATA TTC CTT CTC TAA G3' (SEQ ID NO: 10),

BRCA-2-11F: 5'GAA AAT TCA GCC TTA GC3' (SEQ ID NO: 13),

BRCA-2-11R: 5'ATC AGA ATG GTA GGA AT3' (SEQ ID NO:14),

BRCA-2-11F: 5'TAC AGC AAG TGG AAA GC3' (SEQ ID NO: 17),

BRCA-2-11R: 5'AAG TTT CAG TTT TAC CAA T3' (SEQ ID NO:18),

BRCA-2-11F: 5'ACT TTT TCT GAT GTT CCT GTG3' (SEQ ID NO: 21), or

BRCA-2-11R: 5'TAA AAA TAG TGA TTG GCA ACA3' (SEQ ID NO: 22).

42 ~~68~~ ~~72~~. (Amended) The kit [acording] according to Claim [69] ~~66~~ 40 further comprising at least one container means containing [the pair of oligonucleotide primers of claim 40] a pair of isolated oligonucleotide primers which specifically hybridize to the BRCA2 gene, one of which can effectively hybridize to exon 11 of the BRCA2 gene, and the other can effectively hybridize to either exon 11 or one of the two intron regions flanking exon 11.

REMARKS

The claims have been amended to correct typographical errors, errors in claim numbering, and improper multiple dependencies. No new matter has been introduced by the foregoing amendments.

Favorable consideration and allowance of Claims 1-75 is respectfully requested.

Respectfully submitted,

Rochelle K. Seide
Patent Office Reg. No. 32,300

Attorney for Applicants
(212) 408-2626

Janet M. MacLeod
Patent Office Reg. No. 35,263

Agent for Applicants
(212) 408-2597

BAKER & BOTTS, L.L.P.
30 ROCKEFELLER PLAZA
NEW YORK, NEW YORK 10112-0228
212 705-5000
FACSIMILE 212 705-5020

Amdt. Trans.
PATENT

Our File No.: A31421-2880/2

Date: February 17, 1998

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of : Jennifer L. Lescallet, et al.

Serial No. : 08/984,034 Examiner :

Filed : December 2, 1997 Group Art Unit :

For : CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Transmitted herewith is an amendment in the above-identified application.

1. [X] Small entity status of this application under 37 CFR 1.9 and 1.27 has been established by a verified statement previously submitted.

2. [] A verified statement to establish small entity status under 37 CFR 1.9 and 1.27 is enclosed.

3. [X] No additional fee is required.

CERTIFICATE OF MAILING

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on February 17, 1998.

Attorney Name Janet M. MacLeod Registration No. 35,263

Signature Janet M. MacLeod Date of Signature February 17, 1998

Our Fi. .No. A31421-2880/2

The fee has been calculated as shown below:

	Claims remaining after amendt. (Col. 1)	Highest No. Prev. Paid for (Col. 2)		Present extra (Col. 3)	SMALL ENTITY RATE		FEE	or	OTHER THAN A SMALL ENTITY RATE		FEE
Total	* 79	Minus **79	=	0	x	11 =	$0	or	x	22 =	
Ind.	* 8	Minus ***	= 0		x	41 =	$0	or	x	82 =	
( ) First Presentation of Multiple Dependent Claim					+	135 =		or	+	270 =	
				TOTAL ADDITIONAL FEE		=	$0	or	TOTAL	=	$0

* If the entry in Col. 1 is less than the entry in Col. 2, write "0" in Col. 3.
** If the "Highest No. prev. paid for" in this space is less than 20, write "20" in this space.
*** If the "Highest No. prev. paid for" in this space is less than 3, write "3" in this space.

4.(a)[] An Extension of Time to respond to the PTO communication dated is hereby requested. The required fee, indicated below, is enclosed herewith.

Extension for response (check only one):

	SMALL ENTITY		OTHER THAN A SMALL ENTITY	
Within first month	[]	$ 55	[]	$ 110
Within second month	[]	200	[]	400
Within third month	[]	475	[]	950
Within fourth month	[]	755	[]	1,510

(check and complete the next item, if applicable)

[] An extension for months has already been secured and the fee paid therefor of $ is deducted from the total fee due for the total months of extension now requested.

Extension fee due with this request $.

or

(b)[X] In the event that an extension of time is required, this conditional petition is being made to provide for the possibility that applicant has inadvertently overlooked the need for a petition and fee for extension of time.

5. [] Please charge our Deposit Account No. 02-4377 in the amount of $. Two copies of this sheet are enclosed.

6. [] A check in the amount of $ is attached.

Our File No. A31421-2880/2

7. [X] The Commissioner is hereby authorized to charge payment of any additional filing fees required under 37 CFR 1.16 and/or 37 CFR 1.17 associated with this communication or credit any overpayment to Deposit Account No. 02-4377. Two copies of this sheet are enclosed.

BAKER & BOTTS, L.L.P.

By Janet M. MacLeod
Janet M. MacLeod

PTO Registration No. 35,263

Enclosures

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKETT NO.

EXAMINER	
ART UNIT	PAPER NUMBER
	4

DATE MAILED:

EXAMINER INTERVIEW SUMMARY RECORD

All participants (applicant, applicant's representative, PTO personnel):

(1) Janet MacLeod (3) _____

(2) Lisa Arthur (4) _____

Date of interview 6/10/98

Type: ☒ Telephonic ☐ Personal (copy is given to ☐ applicant ☐ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes ☒ No. If yes, brief description: _____

Agreement ☐ was reached with respect to some or all of the claims in question. ☒ was not reached.

Claims discussed: 1-75

Identification of prior art discussed: —

Description of the general nature of what was agreed to if an agreement was reached, or any other comments: Examiner called to make restriction requirement ~~to~~ Group I: 1-56, 58-75. Group II: 57 drawn to an array. Elected I with traverse.

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

☐ 1. It is not necessary for applicant to provide a separate record of the substance of the interview.

Unless the paragraph below has been checked to indicate to the contrary, A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW (e.g., items 1-7 on the reverse side of this form). If a response to the last Office action has already been filed, then applicant is given one month from this interview date to provide a statement of the substance of the interview.

☐ 2. Since the examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action. Applicant is not relieved from providing a separate record of the substance of the interview unless box 1 above is also checked.

Lisa B. Arthur

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, DC 20231

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/984,034	12/02/97	LESCALLETT, J	A314202880

HM31/0728

BAKER & BOTTS
30 ROCKEFELLER PLAZA
NEW YORK NY 10112

EXAMINER	
ARTHUR, L	

ART UNIT	PAPER NUMBER
1634	5

DATE MAILED: 07/28/98

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Office Action Summary

Application No.	Applicant(s)
08/984,034	Lescallett, J. L., et al.
Examiner	**Group Art Unit**
Lisa Arthur	1634

☐ Responsive to communication(s) filed on _____.

☐ This action is **FINAL.**

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire 3 month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) *1-56 and 58-75* is/are pending in the application.

Of the above, claim(s) 57 is/are withdrawn from consideration.

☐ Claim(s) _____ is/are allowed.

☒ Claim(s) *1-56 and 58-75* is/are rejected.

☐ Claim(s) _____ is/are objected to.

☒ Claims 1-75 are subject to restriction or election requirement.

Application Papers

☒ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on _____ is ☐approved ☐disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number) _____.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____.

☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☒ Notice of References Cited, PTO-892

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____

☒ Interview Summary, PTO-413

☒ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

--- SEE OFFICE ACTION ON THE FOLLOWING PAGES ---

1. This action includes an examination of claims 1-75. The current status of the claims are as follows:

I. Claims 1-56, 58-75 rejected under 35 U.S.C. 112, second paragraph.

II. Claims 1, 23, 24, 25-37, 38-44, 71, 72, 73, 74 are rejected under 35 U.S.C. 102(b).

III. Claims 25-44 rejected under 35 U.S.C. 102(a).

IV. Claim 45-48, 66-70 rejected under 35 U.S.C. 103(a).

Restriction to one of the following inventions is required under 35 U.S.C. 121:

2. I. Claims 1-56, drawn to 58-75, classified in class 536, subclass 24.31 and class 435, subclass 6.

II. Claim 57, drawn to a chip array, classified in class 435, subclass 287.1 .

3. The inventions are distinct, each from the other because:

Inventions I and II are related as process and apparatus for its practice. The inventions are distinct if it can be shown that either: (1) the process as claimed can be practiced by another materially different apparatus or by hand, or (2) the apparatus as claimed can be used to practice another and materially different process. (MPEP § 806.05(e)). In this case the process can be practiced by hand.

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art as shown by their different classifications, restriction for examination purposes as indicated is proper.

4. During a telephone conversation with Janet MacLeod on June 10, 1998 a provisional

election was made with traverse to prosecute the invention of Group I, claims 1-56, 58-75. Affirmation of this election must be made by applicant in replying to this Office action. Claim 57 withdrawn from further consideration by the examiner, 37 CFR 1.142(b), as being drawn to a non-elected invention.

5. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a petition under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(I).

6. Claims 1-56, 58-75 rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

A. Claims 1-24, 66 are indefinite over the recitation of "said fourth oligonucleotide," "said fifth," "said sixth," and "said seventh" because these phrases lack antecedent basis. No "fourth," "fifth," "sixth," "seventh" oligonucleotide was previously referred to. This rejection can be overcome by amending to "said third," "said fourth," "said fifth," and "said sixth" oligonucleotide respectively.

B. Claims 3, 6, 9, 12, 15, 18, 21 are indefinite because it is drawn to a "wild type allele specific oligonucleotide" and depends from claims 2, 5, 8, 11, 14, 17, 20 respectively which are drawn to oligonucleotides that detect the mutant sequence at 2192, 3772, 5193, 5374, 6495,

6909 respectively. Therefore, the claims are unclear as to whether the oligonucleotide is specific for wild type or mutant sequence. The rejection could be overcome by making these claims independent claims.

C. Claims 3, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18, 19, 21, 22, 26-37, 39-44 are indefinite over "complementary" because this language makes the claims unclear as to whether the claimed oligonucleotides are the exact complement, or alternatively, a functional complement, i.e, an oligonucleotide which can be hybridized under any conditions to the recited sequence but is not limited to being a specific length or sequence. This rejection can be overcome by inserting a "the" before "complementary."

D. Claims 5-10, 14-19, and 20-22 are indefinite over "the deletion," (Claims 5-7, 14-19) "the substitution," (Claims 8-10) or "the insertion" (Claims 20-22) because the phrase lacks antecedent basis. The rejection can be overcome by amending to "a deletion" and "a substitution", respectively.

E. Claims 25-37, 69 are indefinite over "a region overlapping the region" because this phrase makes the claims unclear as to what the primer hybridizes to. That is, does the primer hybridize to a portion of the same region as the recited primer sequences or to a different sequence which shares some sequence similarity.

F. Claims 26-37, 39-41, 43, 44 are indefinite over "substantially similar sequence" because the degree of similarity cannot be determined from this phrase and therefore the metes and bounds of the claim cannot be ascertained.

G. Claims 38-46, 48, 68, 70 are indefinite over "effectively hybridize" because it is

unclear whether this term means "specifically" or under any hybridizing condition. This rejection can be overcome by amending "specifically."

H. Claims 49-56 are indefinite because the claims do not recite a positive process step which clearly relates to the preamble. The preamble states that the method is for determining sequence variation in "a gene" but the last step determines variation in BRCA2. This rejection can be overcome by amending the preamble to recite "in the BRCA2 gene" instead of "a gene." Also the claim is indefinite over "predetermined sequence variation" because it's not clear in what the variation is occurring. This rejection can be over come by inserting "in the BRCA2 gene" after "variations."

I. Claim 52 is indefinite over "the allele specific sequence based assay" because it lacks antecedent basis. This phrase is not the same as "an allele specific detection assay." This rejection can be overcome by amending to "the allele specific detection assay."

J. Claims 58, 59, 60, 61 are indefinite over "said DNA fragments" and "said digestion" and "the DNA fragment sequence" (step (a)) because they lack antecedent basis.

K. Claims 62-65 are indefinite over step (d) because it is unclear how a predisposition for cancer is detected by correlating a sequence variation to the presence or absence of the BRCA2 gene.

M. Claim 63 is indefinite over "sequence variation of the BRCA2 gene overlapping the region complementary to . . . " because it is unclear as to what is "overlapping" what and how this relates to a sequence which specifically hybridizes.

N. Claims 71-74 are indefinite over "sequence consisting essentially of " because this

term is only clear with regard compositions and methods but not products since there is no way to determine the part of the structure of the DNA that is not "essential."

O. Claims 71-75 are indefinite over "complementary thereto" because this language makes the claims unclear as to whether the claimed DNA sequence is the exact complement or alternatively a functional complement. This rejection can be overcome by amending to "which is the complement thereof."

7. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless --
>
> (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

8. Claims 1, 23, 24, 25-37, 38-44, 71, 72, 73, 74 are rejected under 35 U.S.C. 102(b) as being anticipated by Tavitigian et al. (Nature Genetics 12: 333-337 (1996)).

Tavitigian et al. teaches an isolated nucleic acid which specifically hybridized to a region containing nucleotide 2192, 1655, 5374, 6495, and 6909 of BRCA2, because they teach the entire gene sequence of BRCA2(Fig. 1). The reference does not specifically teach that the nucleic acid is to be used to detect the recited amino acid change. However, this limitation is an intended use for the oligonucleotide which does not carry patentable weight to distinguish the claimed nucleic acid from that of Tavitigian et al. Tavitigian et al. also teach a labeled BRCA2 nucleic acid (pg.

337, col.1, paragraph 4). Tavitigian et al. also teaches primers which specifically hybridize to exon 11 of BRCA2 (Table 2), which is a region "overlapping" the region where the claimed oligonucleotide hybridize. Because the claims use the term "substantially similar sequences" (see rejection under 35 U.S.C. 112, second paragraph) the primers of the reference anticipate the claims because they can be considered "substantially similar" to hybridize to the same region. Tavitigian et al. also teach primer pairs which amplify exon 11 (Table 2 and pg. 337, col 2, paragraph 3). Tavitigian et al. teach isolated DNA that codes at least part of the BRCA2 which complementary to a BRCA2 gene containing the recited mutations because they teach the wild type BRCA2 which was "complementary" to BRCA2 containing the mutations. Tavitigian et al. teach inserting the BRCA2 DNA into vectors (pg. 337).

9. Claims 25-44 rejected under 35 U.S.C. 102(a) as being anticipated by Panayiotidis et al. (British Journal of Haematology 97: 844-847 (1997)).

Panayiotidis et al. teaches primers and primer pairs to BRCA2 at exon 11 (page 845, col 2-846, col.) which is a region that the primers of SEQ ID No. 1, 2, 5, 6, 9, 10, 13, 14, 17, 18, 21, 22 hybridize to and therefore anticipate the claims.

10. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

11. Claim 45-48, 66-70 rejected under 35 U.S.C. 103(a) as being unpatentable over Tavitigian et al. In views of Mullis et al. (4,683,195).

Tavitigian et al. does not teach labeling the BRCA2 oligonucleotide and primers or packaging the oligonucleotide or primers as a kit. However, Mullis et al. teaches adding a label to primers for PCR in order to detect the amplication product without an additional detection. Mullis et al. also teaches kits containing primers and probes for use in PCR. Therefore, it is prima facie obvious to one of ordinary skill in the art to have added the label to the primers and oligonucletoides of Tavitigian et al. because Mullis et al. taught that labeling the primer allowed easy detection of the amplified product. It also would have been obvious to package the oligonucleotide and primers of Tavitigian et al. as a kit because Mullis et al. teach kits containing oligonucleotides and the ordinary artisan would have been motivated to package the oligonucleotide of Tavitigian et al. as a kit in order to achieve the excepted benefit of more easily marketing, distributing and performing a method and using these oligonucletotides.

12. No claims are allowable but claims 2-22, 49-56, 58-65 and 75 are allowable over the prior art because the art did not teach the specifically recited mutations in BRCA2, and consequently the ordinary artisan would not have been motivated to make oligonucleotide having the recited sequences. The art also does not correlate these mutations to a predisposition for cancer.

13. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Lisa Arthur whose telephone number is (703) 308-3988. The examiner can

normally be reached on Monday-Wednesday from 8:00 to 4:30.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Jones, can be reached on (703) 308-1152. The fax phone number for this Group is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.

Lisa B. Arthur

LISA B. ARTHUR
PRIMARY EXAMINER
GROUP ~~1800~~ 1600
July 27, 1998

TO SEPARATE, HOLD TOP AND BOTTOM EDGES, SNAP-APART AND DISCARD CARBON

FORM PTO-892 (REV. 2-92)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	SERIAL NO.	GROUP ART UNIT	ATTACHMENT TO PAPER NUMBER	
NOTICE OF REFERENCES CITED		08/984,034	1634		5
		APPLICANT(S) Lescallette J.L. et al.			

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
	A	4683195	7/28/87	Mullis et al.	435 ~~536~~	6	
	B						
	C						
	D						
	E						
	F						
	G						
	H						
	I						
	J						
	K						

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHTS. DWG	PERTINENT PP. SPEC.
	L								
	M								
	N								
	O								
	P								
	Q								

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

	R	Panayiotidis P. et al. British Journal of Haematology 97: 849-847 (1997).
	S	Tavitigian et al. Nature Genetics 12: 333-337 (1996).
	T	
	U	

EXAMINER	DATE
Lisa B. Arthur	7/27/98

* A copy of this reference is not being furnished with this office action.
(See Manual of Patent Examining Procedure, section 707.05 (a).)

GAU/1634

BAKER & BOTTS, L.L.P.
30 ROCKEFELLER PLAZA
NEW YORK, NEW YORK 10112-0228
212 705-5000
FACSIMILE 212 705-5020

CPA Trans.
PATENT

CONTINUED PROSECUTION APPLICATION (CPA) REQUEST TRANSMITTAL
(Only for Continuation or Divisional applications under 37 CFR 1.53(d))

Attorney Docket No. 31421-072880.0102

First Named Inventor JENNIFER L. LESCALLET

Express Mail Label No. EJ408151254US

Total Pages _____

January 7, 1999

BY EXPRESS MAIL

Assistant Commissioner for Patents
Box CPA
Washington, DC 20231

Sir:

This is a request for the filing of a

[X] Continuation or [] Divisional application under 37 CFR 1.53(d),
(continued prosecution application (CPA) of prior application No. 08/984,034
filed on Dec. 2, 1997, entitled CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2

The term for response or taking action in the prior application expires on _.

[] An extension of time for response in the prior application is:
[X] filed concurrently herewith.
[] has been filed on_.

[] Enter the unentered amendment previously filed on _____
under 37 CFR 1.116 in the prior nonprovisional application.

[X] A preliminary amendment is enclosed.

[] This application is filed by fewer than all the inventors named in the prior application, 37 CFR 1.53 (d)(4).
[] **DELETE** the following inventor(s) named in the prior nonprovisional application:
_____
_____
[] The inventor(s) to be deleted are set forth on a separate sheet attached hereto.

[] A new power of attorney or authorization of agent (PTO/SB/81) is enclosed.

CPA Trans.
PATENT

Attorney Docket No. A31421-072880.0102

Information Disclosure Statement (IDS) is enclosed.

[] PTO-1449

[] Copies of IDS Citations

Small entity status:

[] A small entity statement is enclosed.

[X] A small entity statement was filed in the prior nonprovisional application and such status is still proper and desired.

[] is no longer claimed.

The filing fee has been calculated as shown below:

FOR	(Col. 1) No.Filed		(Col. 2)* No. Extra	Small Entity Rate	Fee	OR	Other Than A Small Entity Rate	Fee
Basic Fee					$380			$760
Total Claims	72	-20=	52	x $9=	$468		x $18 =	$
Ind. Claims	7	-3 =	4	x $39 =	$156		x $78 =	$
Multiple Dependent Claim				+ $130 =	$		+$260=	$
				Total	$1,004			$

* If the difference in Col. 1 is less than zero, enter "0" in Col. 2.

Calculation of extension fee (37 CFR 1.17(1)-(5)):

	Extension (months)	Fee for other than small entity	Fee for small entity
[ ]	one month	$ 110.00	$ 55.00
[ ]	two months	$ 380.00	$190.00
[X]	three months	$ 870.00	$435.00
[ ]	four months	$1,360.00	$680.00
[ ]	five months	$1,850.00	$925.00

CPA Trans.
PATENT

Attorney Docket No. A31421-072880.0102

Fee $ _

[X] If an additional extension of time is required, please consider this a petition therefor.

(check and complete the next item, if applicable)

[ ] An extension for months has already been secured and the fee paid therefor of $ is deducted from the total fee due for the total months of extension now requested.

Extension fee due with this request $ _

Fee Payment Being Made:

[X] Enclosed

[X]	Basic filing fee	$1004.00
	Extension of Time Fee	$435.00
	Total Fees Enclosed	$1,439.00

[X] A check in the amount of $1439.00 is enclosed.

[] Other _____

[X] The Commissioner is hereby authorized to charge payment of any additional filing fees required under 37 CFR 1.16, 1.17, and 1.21(h) associated with this communication or credit any overpayment to Deposit Account No. 02-4377. Two copies of this sheet are enclosed.

BAKER & BOTTS, L.L.P.

By Janet M. MacLeod

Janet M. MacLeod

PTO Registration No. 35,263

Enclosures

FILE NO. A31421-072880.0102

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Jennifer L. Lescallet, et al.	
Serial No.	:	08/984,034	Examiner:
Filed	:	December 2, 1997	Group Art Unit:
For	:	CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2	

PRELIMINARY AMENDMENT

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

January 7, 1999
Date of Deposit

Janet M. MacLeod
Agent Name

35,263
PTO Registration No.

Signature

January 7, 1999
Date of Signature

Assistant Commissioner for Patents

Washington, D.C. 20231

Sir:

Prior to examination of the above-identified application, please amend the claims as follows:

IN THE CLAIMS:

Please cancel Claims 71-73 without prejudice.

Claims 3, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18, 19, 21 and 22, line 3, after "or" insert --the--.

Claims 26-37, line 4, delete "a" and substitute therefor --the--.

Claims 39-44, line 6, delete "a" and substitute therefor --the--.

Claims 5, 8, 14, 17 and 20, line 2, delete the second "the" and substitute therefor --a--.

Claims 38, lines 3 and 4, delete "effectively" and substitute therefor --specifically--.

Claim 49, line 2, delete "a gene sample" and substitute therefor --the BRCA2 gene--.

Claim 49, line 5, after "variations" insert --in the BRCA2 gene--.

Claim 52, line 2, delete "sequence-based" and substitute therefor --detection--.

(N.E. Claims cancelled)

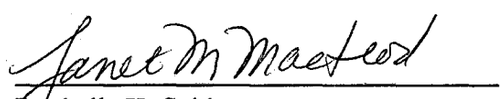
Claims 71 and 73, line 5, delete "complementary thereto" and substitute therefor --which is the complement thereof--.

REMARKS

The claims have been amended to further define the subject matter of the invention. No new matter has been added.

Favorable consideration and allowance of Claims 1-70, 74 and 75 is respectfully requested.

Respectfully submitted,



Rochelle K. Seide
Patent Office Reg. No. 32,300

Attorney for Applicants
(212) 408-2626

Janet M. MacLeod
Patent Office Reg. No. 35,263

Agent for Applicants
(212) 408-2597

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

DW

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/984,034	12/02/97	LESCALLETT J	A314202880

HM22/0318

BAKER & BOTTS
30 ROCKEFELLER PLAZA
NEW YORK NY 10112

EXAMINER	
ARTHUR, L	
ART UNIT	**PAPER NUMBER**
1634	8

DATE MAILED: 03/18/99

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Office Action Summary	Application No. 08/984,034	Applicant(s) Lescallett Eet Al.
	Examiner First Last	Group Art Unit 1234

☒ Responsive to communication(s) filed on *Jan 7, 1999*.

☐ This action is **FINAL.**

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire *3* month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) *1-70, 74, and 75* is/are pending in the application.

Of the above, claim(s) *57* is/are withdrawn from consideration.

☒ Claim(s) *45-56* is/are allowed.

☒ Claim(s) *1-44, 58-70, 74, and 75* is/are rejected.

☐ Claim(s) _____ is/are objected to.

☐ Claims _____ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on _____ is ☐approved ☐disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number) _____.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____.

☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☐ Notice of References Cited, PTO-892

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____

☐ Interview Summary, PTO-413

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

--- SEE OFFICE ACTION ON THE FOLLOWING PAGES ---

1. This application is a Continuing Prosecution Application filed under 37 CFR 1.53(d) on January 7, 1999. Claims 1-70 and 74-75 are pending but claim 57 has been withdrawn by the restriction requirement made in the June 28, 1998 office action.

2. The amendment to claims 71 and 73 made at the bottom of the preliminary amendment filed January 7, 1999 was not entered because these claims have been canceled.

3. Claim 74 is objected because it depends from a canceled claim, i.e. claim 73. Correction is required.

4. Claims 1-45,47 and 58-75 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

A) Claims 1-24,66-70 are indefinite over the recitation of "said fourth oligonucleotide", said fifth oligonucleotide",'said "sixth oligonucleotide", "said seventh oligonucleotide" because these phrases lack antecedent basis. No fourth, fifth, sixth, seventh oligonucleotides were previously referred to. This rejection can be overcome by amending to "Said third oligonucleotide", "said fourth oligonucleotide", said fifth oligonucleotide", and "said "sixth oligonucleotide".

B) Claims 3,6,9,12,15,18 and 21 are indefinite because they are drawn to a "wild type allele specific oligonucleotide" and depend from claims 2,5,8,11,14,17 and 20, respectively, which are drawn to oligonucleotides that detect the mutant sequence at 2192, 3772,5193, 5374,6495 and 6909, respectively. Therefore, the claims are unclear as to whether the oligonucleotides are specific for wild type or for mutant sequences. The rejection could be overcome by writing these claims as independent claims.

C) Claims 25-37, 47and 67 are indefinite over the recitation of "a region overlapping the region" because this phrase makes the claims unclear as to what the primer hybridizes to. That is, the claims are not clear as to whether the primers hybridize to a portion of the same region as the recited primer sequence or to a different sequence which shares some sequence similarity.

D) Claims 26-37,39-44 are indefinite over the recitation of "substantially similar sequence" because the degree of similarity cannot be determined from this phrase and therefore the metes and bounds of the claims cannot be ascertained.

E) Claims 58,59,60 and 61 are indefinite over "said DNA fragments and "said digestion" and "the DNA fragment sequence (step(a)) because they lack antecedent basis.

F) Claims 62-65 are indefinite over step(d) because it is unclear how a predisposition for cancer is detected by correlating a sequence variation to the presence or absence of the BRCA2 gene.

G) Claim 63 is indefinite over the recitation of "sequence variation of the BRCA2 gene overlapping the region complementary to..." because it is unclear as to what is "overlapping" what and how this relates to a sequence which specifically hybridizes.

H) Claims 74 and 75 are indefinite over the recitation of "complementary thereto" because this language makes the claims unclear as to whether the claimed DNA sequence is the exact complement or alternatively a functional complement. This rejection ca be overcome by amending to "which is the complement thereof".

5. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless --
>
> (a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or for sale in this country, more than one year prior to the date of application for patent in the United States.

6. Claims 1,23,24,25-37,38-44 and 74 are rejected under 35 U.S.C. 102(b) as being anticipated by Tavitigian et al. (Nature Genetics 12: 333-337 (1996)..

Tavitigian et al. Teach an isolated nucleic acid which specifically hybridized to a region containing nucleotide 2192,1655,5374,6495 and 6909 of BRCA2 because they teach the entire gene sequence of BRCA2 (figure 1). The reference does not specifically teach that the nucleic acid is to be used to detect the recited amino acid changes. However, this limitation is an intended use for the oligonucleotide which does not carry patentable weight to distinguish the

claimed nucleic acids from that of Tavitigian et al. Tavitigian et al. Also taught a labeled BRCA2 nucleic acid (page 337, column 1, paragraph 4). Tavitigian et al. Also taught primers which specifically hybridized to exon 11 of BRCA2 (Table 2) which is a region "overlapping" the region where the claimed oligonucleotides hybridize. Because the claims use the term "substantially similar sequences" (see the rejection made under 35 U.S.C. 112, second paragraph), the primers of the reference anticipate the claimed because the primers of Tavitigian et al. Are "substantially similar" since they hybridize to the same region. Tavitigian et al. Also taught primer pairs which amplify exon 11 (Table 2 and page 337, column 2, paragraph 3). Tavitigian et al. Taught isolated DNA that codes at least part of the BRCA2 gene which is complementary to a BRCA2 gene containing the recited mutations because they teach the wild type BRCA2 which was "complementary to BRCA2 containing the mutations. Tavitigian et al also taught inserting the BRCA2 DNA into vectors (page 337).

7. Claims 25-44 are rejected under 35 U.S.C. 102(a) as being anticipated by Panayiotidis et al (British J. Haematology 97:844-847 (1997)).

Panayiotidis et al. Teach primers and primer pairs to BRCA2 at exon 11 (page 849, column 2 through page 846) which is a region that the primers of the instant claims having SEQ ID Nos 1,2,5,6,9,10,13,14,17,18,21 and 22 hybridize to. Therefore, Panayiotidis et al anticipate the claims as written.

8. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102.of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103© and potential 35 U.S.C. 102(f) or (g) prior art under 35 U.S.C. 103(a).

9. Claims 45-48 and 66-70 are rejected under 35 U.S.C. 103(a) as being unpatentable over Tavitigian et al. In view of Mullis (4,683,195).

Tavitigian et al. Teach an isolated nucleic acid which specifically hybridized to a region containing nucleotide 2192,1655,5374,6495 and 6909 of BRCA2 because they teach the entire gene sequence of BRCA2 (figure 1). The reference does not specifically teach that the nucleic acid is to be used to detect the recited amino acid changes. However, this limitation is an intended use for the oligonucleotide which does not carry patentable weight to distinguish the claimed nuclei acids from that of Tavitigian et al. Tavitigian et al. Also taught a labeled BRCA2 nucleic acid (page 337, column 1, paragraph 4). Tavitigian et al. Also taught primers which

specifically hybridized to exon 11 of BRCA2 (Table 2) which is a region "overlapping" the region where the claimed oligonucleotides hybridize. Because the claims use the term "substantially similar sequences" (see the rejection made under 35 U.S.C. 112, second paragraph), the primers of the reference anticipate the claimed because the primers of Tavitigian et al. Are "substantially similar" since they hybridize to the same region. Tavitigian et al. Also taught primer pairs which amplify exon 11 (Table 2 and page 337, column 2, paragraph 3). Tavitigian et al. Taught isolated DNA that codes at least part of the BRCA2 gene which is complementary to a BRCA2 gene containing the recited mutations because they teach the wild type BRCA2 which was "complementary to BRCA2 containing the mutations. Tavitigian et al also taught inserting the BRCA2 DNA into vectors (page 337).

Tavitigian et al does not teach labeling the BRCA2 oligonucleotides and primers or packaging them as a kit.

However, Mullis et al taught adding a label to primers for PCR to allow detection of the amplified product without having to perform an additional detection step. Mullis et al also taught kits containing primers and probes for use in PCR

Therefore, it would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to have added the label to the primers and oligonucleotides of Tavitigian et al. Because Mullis et al. Taught that labeling the primer allowed easy detection of the amplified product. It also would have been prima facie obvious to have packaged the oligonucleotides and primers of Tavitigian et al. As a kit because Mullis et al. Taught kits

containing oligonucleotides and the ordinary artisan would have been motivated to package the oligonucleotides of Tavitigian et al. As a kit in order to have achieved the expected benefit of more easily marketing, distributing, and performing the method of Tavitigian.

10.Claims 49-56 are allowable and claims 2-22 58-95 and 75 are allowable over the prior art because the art did not teach the specifically recited mutations in the BRCA2 gene, and consequently, the ordinary artisan would not have been motivated to make oligonucleotides having the recited sequences. The art also did not correlate these mutations to a predisposition to cancer.

11. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Lisa Arthur whose telephone number is (703) 308-3988. The examiner can normally be reached on Monday-Wednesday from 7:00AM to 3:00PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Jones, can be reached on (703) 308-1152. The fax phone number for the organization where this application or proceeding is assigned is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-0196.

Lisa B. Arthur

LISA B. ARTHUR
PRIMARY EXAMINER
GROUP 1800

March 16, 1999

BAKER & BOTTS, L.L.P.
30 ROCKEFELLER PLAZA
NEW YORK, NEW YORK 10112-0228
212 705-5000
FACSIMILE 212 705-5020

Amdt. Trans. PATENT

Our File No.: A31421-072880.0102

Date: September 20, 1999

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :	Jennifer L. Lescallet, et al.			
Serial No.	: 08/984,034	Examiner	: Arthur, L.	
Filed	: December 2, 1997	Group Art Unit	: 1634	
For	: CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2			

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Transmitted herewith is an amendment in the above-identified application.

1. [X] Small entity status of this application under 37 CFR 1.9 and 1.27 has been established by a verified statement previously submitted.

2. [] A verified statement to establish small entity status under 37 CFR 1.9 and 1.27 is enclosed.

3. [] No additional fee is required.

CERTIFICATE OF MAILING

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on September 20, 1999.

Attorney Name Janet M. MacLeod

Registration No. 35,263

Signature Janet M MacLeod

Date of Signature: September 20, 1999

Our File No. A31421-072880.0102

The fee has been calculated as shown below:

		Claims remaining after amendt. (Col. 1)		Highest No. Prev. Paid for (Col. 2)		Present extra (Col. 3)	SMALL ENTITY RATE		FEE	or	OTHER THAN A SMALL ENTITY RATE		FEE
Total	*	71	Minus	**72	=	0	x	9 =	$0	or	x	18 =	
Ind.	*	18	Minus	***7	=	11	x	39 =	$429	or	x	78 =	
( ) First Presentation of Multiple Dependent Claim							+	130 =	$0	or	+	260 =	
						TOTAL ADDITIONAL FEE		=	$429	or	TOTAL	=	

* If the entry in Col. 1 is less than the entry in Col. 2, write "0" in Col. 3.
** If the "Highest No. prev. paid for" in this space is less than 20, write "20" in this space.
*** If the "Highest No. prev. paid for" in this space is less than 3, write "3" in this space.

4.(a)[X] An Extension of Time to respond to the PTO communication dated March 18, 1999 is hereby requested. The required fee, indicated below, is enclosed herewith.

Extension for response (check only one):

	SMALL ENTITY		OTHER THAN A SMALL ENTITY	
Within first month	[]	$ 55	[]	$ 110
Within second month	[]	190	[]	380
Within third month	[X]	435	[]	870
Within fourth month	[]	680	[]	1,360

(check and complete the next item, if applicable)

[] An extension for months has already been secured and the fee paid therefor of $ is deducted from the total fee due for the total months of extension now requested.

Extension fee due with this request $.

or

(b)[X] In the event that an extension of time is required, this conditional petition is being made to provide for the possibility that applicant has inadvertently overlooked the need for a petition and fee for extension of time.

5. [] Please charge our Deposit Account No. 02-4377 in the amount of $. Two copies of this sheet are enclosed.

Our File No. A31421-072880.0102

6. [X] A check in the amount of $864 is attached.

7. [X] The Commissioner is hereby authorized to charge payment of any additional filing fees required under 37 CFR 1.16 and/or 37 CFR 1.17 associated with this communication or credit any overpayment to Deposit Account No. 02-4377. Two copies of this sheet are enclosed.

BAKER & BOTTS, L.L.P.

By Janet M. MacLeod
Janet M. MacLeod

PTO Registration No. 35,263

Enclosures

FILE NO. A31421-072880.0102

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Jennifer L. Lescallet, et al.	
Serial No.	:	08/984,034	Examiner: Arthur, L.
Filed	:	December 2, 1997	Group Art Unit: 1634
For	:	CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2	

AMENDMENT

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231

September 20, 1999
Date of Deposit

Janet M. MacLeod
Attorney Name

35,263
PTO Registration No.

Janet M. MacLeod
Signature

September 20, 1999
Date of Signature

Assistant Commissioner for Patents

Washington, D.C. 20231

Sir:

This paper is in response to the Office Action mailed March 18, 1999 for the above-identified application. Applicants request a three-month extension of time for responding to the Office Action, and enclose a check in the amount of $435 in

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02 FC:202 429.00 OP

payment of the fee under 37 C.F.R. § 1.17(a). Please amend the claims as follows and consider the following remarks.

IN THE CLAIMS:

Please cancel Claims 1, 11-13, 39-44, 57, 72 and 74 without prejudice.

Please amend the claims as follows:

Claim 2, line 1, delete "The" and substitute therefor --An--; delete "according to Claim 1";

line 3, after "2192" insert --of a BRCA2 gene--.

Claim 3, line 1, ~~delete~~ "wildtype allele specific";

line 2, delete "according to claim 2".

Claim 4, line 1, ~~delete~~ "The" and substitute therefor --An--; delete "mutant allele specific".

Claim 5, line 1, delete "according to claim 1";

line 3, ~~after~~ "3772" insert --of a BRCA2 gene--.

Claim 6, line 1, ~~delete~~ "wildtype allele specific";

line 2, delete "according to claim 5".

Claim 7, line 1, delete "mutant allele specific".

Claim 8, line 1, delete "The" and substitute therefor --An--; delete "according to claim 1";

line 3, after "5193" insert --of a BRCA2 gene--.

Claim 9, line 1, delete "wildtype allele specific";

line 2, delete "according to claim 8".

Claim 10, line 1, delete "mutant allele specific".

Claim 14, line 1, delete "The" and substitute therefor --An--; delete "according to claim 1";

line 3, after "5374" insert --of a BRCA2 gene--.

Claim 15, line 1, delete "wildtype allele specific";

line 2, delete "according to claim 14".

Claim 16, line 1, delete "mutant allele specific".

Claim 17, line 1, delete "The" and substitute therefor --An--; delete "according to claim 1";

line 3, after "6495" insert --of a BRCA2 gene--.

Claim 18, line 1, delete "wildtype allele specific";

line 2, delete "according to claim 17".

Claim 19, line 1, delete "mutant allele specific".

Claim 20, line 1, delete "The" and substitute therefor --An--; delete "according to claim 1";

line 3, after "6909" insert --of a BRCA2 gene--.

Claim 21, line 1, delete "wildtype allele specific";

line 2, delete "according to claim 20".

Claim 22, line 1, delete "mutant allele specific".

Claim 23, line 1, delete "claim 1" and substitute therefor --any one of Claims 2, 5, 8, 14, 17 and 20--.

Claim 25, line 2, after "BRCA2" insert --gene--;

lines 3-5, delete "hybridizing to the BRCA2 gene at a region overlapping the region where one of the following oligonucleotides hybridizes" and substitute therefor --comprising the sequence--.

Claims 26-37, lines 3-5, delete "or a substantially similar sequence capable of hybridizing to the complementary polynucleotide and initiating DNA synthesis along the complementary polynucleotide".

Claim 48, line 1, delete "45" and substitute therefor --47--.

Claim 58, line 3, delete "said" and substitute therefor --an--; delete "into" and substitute therefor --to obtain DNA fragments--;

lines 4-5, delete "obtained from said digestion";

line 11, before "DNA" insert --sequence of said--; after "fragment" delete "sequence".

Claim 60, line 1, before "sequence" insert --DNA fragment containing the --.

Claim 63, line 13, after "23" insert --or--.

lines 14-18, delete ", or a sequence capable of specific hybridization to a region on the BRCA2 gene or said sequence variation of the BRCA2 gene overlapping the region complementary to SEQ ID NO: 3, 4, 7, 8, 11, 12, 15, 16, 19, 20, 23 or 24".

Claim 66, line 5, delete "Claim 1" and substitute therefor --any one of Claims 2, 5, 8, 14, 17, 20--.

Claim 67, lines 4-7, delete "capable of hybridizing to a BRCA2 and initiating DNA synthesis to extend the primer, said isolated oligonucleotide primer hybridizing to the BRCA2 gene at a region overlapping the region where one of the following oligonucleotides hybridizes" and substitute therefor --comprising the sequence--.

Claim 75, line 6, delete "complementary thereto" and substitute therefor --which is the complement thereof--.

21 38. (Twice Amended) A pair of isolated oligonucleotide primers which specifically hybridize to the BRCA2 gene, [one of which can specifically hybridize to exon 11 of the BRCA2 gene, and the other can specifically hybridize to either exon 11 or one of the two intron regions flanking exon 11] said pair of primers selected from the group consisting of:

BRCA-2-11F: 5'TGG TAC TTT AAT TTT GTC ACT T3' (SEQ ID NO:1), and
BRCA-2-11R: 5'TGC AGG CAT GAC AGA GAA T3' (SEQ ID NO: 2);

BRCA-2-11F: 5'CTC AGA TGT TAT TTT CAA AGC3' (SEQ ID NO: 5); and
BRCA-2-11R: 5'CTG TTA AAT AAC CAG AAG CAC3' (SEQ ID NO: 6);

C1

BRCA-2-11F: 5'GCA AAG ACC CTA AAG TAC AG3' (SEQ ID NO: 9), and
BRCA-2-11R: 5'CAT CAA ATA TTC CTT CTC TAA G3' (SEQ ID NO: 10);

BRCA-2-11F: 5'GAA AAT TCA GCC TTA GC3' (SEQ ID NO: 13), and
BRCA-2-11R: 5'ATC AGA ATG GTA GGA AT3' (SEQ ID NO: 14);

BRCA-2-11F: 5'TAC AGC AAG TGG AAA GC3' (SEQ ID NO: 17), and
BRCA-2-11R: 5'AAG TTT CAG TTT TAC CAA T3' (SEQ ID NO: 18); and

C1

BRCA-2-11F:5'ACT TTT TCT GAT GTT CCT GTG3' (SEQ ID NO: 21), and

BRCA-2-11R: 5'TAA AAA TAG TGA TTG GCA ACA3' (SEQ ID NO: 22).

36 ~~62~~. (Amended) A method of detecting a predisposition or higher susceptibility to cancer in an individual, comprising:

(a) digesting DNA from said individual to obtain DNA fragments,

(b) separating said DNA fragments obtained from said digestion,

(c) subjecting said DNA fragments to hybridization [hybridizing a DNA fragment] with an allele specific oligonucleotide having a nucleotide sequence capable of

D specifically

C2

D

hybridizing to [either] ~~a polynucleotide having a sequence contained within the BRCA2 gene sequence, or~~ a polynucleotide having a sequence variation at nucleotide number 2192, 3772, 5193, 5374, 6495 or 6909 of the BRCA2 gene sequence, thereby determining the absence or presence of said sequence variation in the BRCA2 gene of said individual, and

(d) correlating the presence [or absence] of said sequence variation with [the respective presence or absence of the BRCA2 gene, thereby determining] a predisposition or higher susceptibility to cancer.

REMARKS

In the Office Action mailed March 18, 1999, Claim 74 has been objected to as depending from a canceled claim. Claim 1-45, 47 and 58-75 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. Claims 1, 23, 24, 25-37, 38-44 and 74 have been rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Tavitigian et al. (1996) Nature Genetics 12:333 ("Tavitigian et al."). Claims 25-44 have been rejected under 35 U.S.C. § 102(a) as allegedly anticipated by Panayiotidis et al. (1997) British J. Haematology 97:844 ("Panayotidis et al."). Claims 45-48 and 66-70 have been rejected under 35 U.S.C. § 103(a) as allegedly rendered obvious by Tavitigian et al. in view of U.S. Patent No. 4,683,195 to Mullis ("Mullis"). Claims 49-56 have been indicated to be allowable. In view of the foregoing objections and rejections, Applicants have amended the claims and provide the following comments.

Claim 57 has been canceled without prejudice. Applicants reserve the right to file a divisional application directed to the subject matter of Claim 57.

Claim 74 has been objected to as depending from a canceled claim. In the interest of advancing prosecution, Claim 74 has been canceled without prejudice.

Claims 1-45, 47 and 58-75 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite. In particular, Claims 1-24 and 66-70 are

allegedly indefinite in the recitations "said fourth oligonucleotide", "said fifth nucleotide", and so on. In the interest of advancing prosecution, Claims 1 and 11-13 have been canceled without prejudice. Claims 2, 5, 8, 11, 14, 17 and 20 have been amended to independent form. Claim 66 has been amended to depend upon Claim 2, 5, 8, 11, 14, 17 or 20. The remaining rejected claims depend upon the amended claims.

Claims 3, 6, 9, 12, 15, 18 and 21 are allegedly indefinite in the recitation "wild-type" allele specific oligonucleotide. In accordance with the Examiner's suggestion, the claims have been rewritten in independent form. Claim 12 has been canceled without prejudice.

Claims 25-37, 47 and 67 are allegedly indefinite in the recitation "a region overlapping the region". Claim 25 has been amended to delete the term "overlapping the region". The remaining rejected claims depend upon amended Claim 25.

Claims 26-37 and 39-44 are allegedly indefinite in the recitation "substantially similar sequence". Claims 26-37 and 39-44 have been amended to delete "substantially similar".

Claims 58, 59, 60 and 61 are allegedly indefinite for lacking antecedent basis for various terms. Claims 58 and 60 have been amended to provide antecedent basis.

Claims 62-65 are allegedly indefinite in step (d). Claim 62 has been amended to clarify that the presence of a sequence variation in the BRCA2 gene is correlated with a predisposition or higher susceptibility to cancer.

Claim 63 is allegedly indefinite in the recitation "overlapping". In the interest of advancing prosecution, Claim 63 has been amended to delete the allegedly indefinite language.

Claims 74 and 75 are allegedly indefinite in the recitation "complementary thereto". Claim 74 has been canceled without prejudice. Claim 75 has been amended in accordance with the Examiner's suggestion.

In view of the foregoing comments and amendments, withdrawal of the rejection of Claims 1-45, 47 and 58-75 is respectfully requested.

Claims 1, 23-44 and 74 have been rejected under 35 U.S.C. § 102(b) as allegedly anticipated by Tavitigian et al. The Examiner has alleged that the entire gene sequence of BRCA2 disclosed by Tavitigian et al. anticipates the isolated nucleic acids of the present invention. Further, the Examiner has alleged that Tavitigian et al. teach primers that amplify exon 11 of the BRCA2 gene.

In the interest of advancing prosecution, Claim 1 has been canceled without prejudice. Claim 23 has been amended to depend upon Claims 2, 5, 8, 14, 17 and

20. Claims 25-37 have been amended to delete "substantially similar sequences". Claim 38 has been amended to incorporate the subject matter of Claims 39-44. Claims 39-44 and 74 have been canceled without prejudice. The subject matter of the amended claims is not taught or suggested by Tavitigian et al. Withdrawal of the rejection of Claims 1, 23-44 and 74 under 35 U.S.C. § 102(b) is respectfully requested.

Claims 25-44 have been rejected under 35 U.S.C. § 102(a) as allegedly anticipated by Panayiotidis et al. The Examiner has alleged that Panayiotidis et al. teach primers and primer pairs to BRCA2 at exon 11.

In the interest of advancing prosecution, Claims 25-38 have been amended as discussed herein above, and Claims 39-44 have been canceled without prejudice. The subject matter of the amended claims is not taught or suggested by Panayiotidis et al. Withdrawal of the rejection under 35 U.S.C. § 102(a) is respectfully requested.

Claims 45-48 and 66-70 have been rejected under 35 U.S.C. § 103(a) as allegedly rendered obvious by Tavitigian et al. in view of Mullis. The Examiner has applied Tavitigian et al. as described above, and has alleged that it would have been obvious to label primers and package primers as a kit as allegedly taught by Mullis.

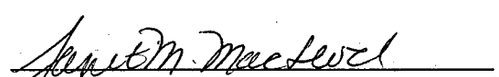
Claims 45-48 depend upon Claims 38 and 25, which have been amended hereinabove. Claim 66 depends on Claims 2, 5, 8, 11, 14, 17 and 20, which have also

been amended as discussed above. Claim 67 has been amended to recite specific primers. Claim 68 has been canceled without prejudice. Claims 69 and 70 depend upon amended Claims 25 and 38, respectively. The subject matter of the amended claims is not taught or suggested by the combination of Tavitigian et al. and Mullis. Accordingly, withdrawal of the rejection of Claims 45-48 and 66-70 under 35 U.S.C. § 103(a) is respectfully requested.

Claims 49-56 have been indicated to be allowable. Claims 2-22, 58-65 and 75 have been indicated to be allowable over the prior art. As acknowledged by the Examiner, the prior art did not teach the specifically recited mutations of the BRCA 2, nor did the art correlate the mutations to a predisposition to cancer. Further, one would not have been motivated to make oligonucleotides to the recited sequences.

In view of the foregoing remarks and amendments, favorable reconsideration of Claims 2-10, 14-38, 58-70 and 75, and allowance of Claims 2-10, 14-38, 49-56, 58-70 and 75 is respectfully requested.

Respectfully submitted,



Rochelle K. Seide
Patent Office Reg. No. 32,300

Janet M. MacLeod
Patent Office Reg. No. 35,263

Attorneys for Applicants
(212) 408-2597

Notice of Allowability

Application No.	Applicant(s)
08/984,034	Lescallett et al.
Examiner	**Group Art Unit**
Lisa Athur	1655

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance and Issue Fee Due or other appropriate communication will be mailed in due course.

☒ This communication is responsive to *September 22, 1999*

☒ The allowed claim(s) is/are *2-10, 14-24, 38, 45, 46, 49-56, 58-68, 70, and 75*

☐ The drawings filed on _____ are acceptable.

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number) _____.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____

☒ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

A SHORTENED STATUTORY PERIOD FOR RESPONSE to comply with the requirements noted below is set to EXPIRE **THREE MONTHS** FROM THE "DATE MAILED" of this Office action. Failure to timely comply will result in ABANDONMENT of this application. Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

☐ Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL APPLICATION, PTO-152, which discloses that the oath or declaration is deficient. A SUBSTITUTE OATH OR DECLARATION IS REQUIRED.

☐ Applicant MUST submit NEW FORMAL DRAWINGS

☐ because the originally filed drawings were declared by applicant to be informal.

☐ including changes required by the Notice of Draftsperson's Patent Drawing Review, PTO-948, attached hereto or to Paper No. _____.

☐ including changes required by the proposed drawing correction filed on _____, which has been approved by the examiner.

☐ including changes required by the attached Examiner's Amendment/Comment.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the reverse side of the drawings. The drawings should be filed as a separate paper with a transmittal lettter addressed to the Official Draftsperson.

☐ Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Any response to this letter should include, in the upper right hand corner, the APPLICATION NUMBER (SERIES CODE/SERIAL NUMBER). If applicant has received a Notice of Allowance and Issue Fee Due, the ISSUE BATCH NUMBER and DATE of the NOTICE OF ALLOWANCE should also be included.

Attachment(s)

☐ Notice of References Cited, PTO-892

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

☐ Interview Summary, PTO-413

☒ Examiner's Amendment/Comment

☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material

☐ Examiner's Statement of Reasons for Allowance

11/D Amur
L. Amur
12/20/99

EXAMINER'S AMENDMENT

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Janet MacLeod on December 16, 1999.

Amend the claims as follows:

I. Cancel claims 25-37, 47, 48, 69.

II. In claims 2,3,5,6,8,9,11,12,14,15,17 and 18, line 3, delete the "s" at the end of "oligonucleotides".

III. In claim 36, line 7, insert --specifically-- before "hybridizing" and delete the phrase "a polynucleotide having a sequence contained within the BRCA2 gene sequence or"

IV. In claim 49, on line 2, after "gene", insert --at nucleotide number 2192, 3772, 5793, 5374, 6495 or 6909--, and on line 4, delete "predetermined" and replace with --of said--.

V. In claim 50, line 2, delete "predetermined" and replace with --of said--.

VI. In claim 53, delete "the predetermined", replace with --said--.

VII. In claim 58, step (d), line 2, delete "for" and replace with --to determine-- and insert -- or absence-- after "presence".

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Lisa Arthur whose telephone number is (703) 308-3988. The examiner can normally be reached on Monday-Thursday from 7:00 am to 1:00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Gary Jones, can be reached on (703) 308-1152. The fax phone number for the organization where this application or proceeding is assigned is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-0196.

Lisa B. Arthur

LISA B. ARTHUR
PRIMARY EXAMINER
GROUP ~~1800~~ 1600

December 20, 1999

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

NOTICE OF ALLOWANCE AND ISSUE FEE DUE

HM22/1221

BAKER & BOTTS
30 ROCKEFELLER PLAZA
NEW YORK NY 10112

APPLICATION NO.	FILING DATE	TOTAL CLAIMS	EXAMINER AND GROUP ART UNIT	DATE MAILED
08/984,034	12/02/97	044	ARTHUR, L 1655	12/21/99
First Named Applicant	LESCALLETT,		35 USC 154(b) term ext. = 0 Days.	

TITLE OF INVENTION: CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2

ATTY'S DOCKET NO.	CLASS-SUBCLASS	BATCH NO.	APPLN. TYPE	SMALL ENTITY	FEE DUE	DATE DUE
1 A314202880	435-006.000	G05	UTILITY	YES	$605.00	03/21/00

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED.

THE ISSUE FEE MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED.

HOW TO RESPOND TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.
If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is changed, pay twice the amount of the FEE DUE shown above and notify the Patent and Trademark Office of the change in status, or
B. If the status is the same, pay the FEE DUE shown above.

If the SMALL ENTITY is shown as NO:

A. Pay FEE DUE shown above, or

B. File verified statement of Small Entity Status before, or with, payment of 1/2 the FEE DUE shown above.

II. Part B-Issue Fee Transmittal should be completed and returned to the Patent and Trademark Office (PTO) with your ISSUE FEE. Even if the ISSUE FEE has already been paid by charge to deposit account, Part B Issue Fee Transmittal should be completed and returned. If you are charging the ISSUE FEE to your deposit account, section "4b" of Part B-Issue Fee Transmittal should be completed and an extra copy of the form should be submitted.

III. All communications regarding this application must give application number and batch number.
Please direct all communications prior to issuance to Box ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PATENT AND TRADEMARK OFFICE COPY

PART B—ISSUE FEE TRANSMITTAL

Complete and mail this form, together with applicable fees, to: **Box ISSUE FEE**
Assistant Commissioner for Patents
Washington, D.C. 20231

BH

PD

MAILING INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE. Blocks 1 through 4 should be completed where appropriate. All further correspondence including the Issue Fee Receipt, the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Legibly mark-up with any corrections or use Block 1)

[illegible]1221

BAKER & BOTTS
30 ROCKEFELLER PLAZA
NEW YORK NY 10112

OIPE JC90 MAR 13 2000 PATENT & TRADEMARK OFFICE

Note: The certificate of mailing below can only be used for domestic mailings of the Issue Fee Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing.

Certificate of Mailing

I hereby certify that this Issue Fee Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Box Issue Fee address above on the date indicated below.

Janet M. MacLeod (Depositor's name)

Janet M. MacLeod (Signature)

March 6, 2000 (Date)

APPLICATION NO.	FILING DATE	TOTAL CLAIMS	EXAMINER AND GROUP ART UNIT	DATE MAILED
08/984,034	12/02/97	044	ARTHUR, L 1655	12/21/99

First Named Applicant: LESCALLETT, 35 USC 154(b) term ext. = 0 Days.

TITLE OF INVENTION: CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2

ATTY'S DOCKET NO.	CLASS-SUBCLASS	BATCH NO.	APPLN. TYPE	SMALL ENTITY	FEE DUE	DATE DUE
1 A314202880	435-006.000	G05	UTILITY	YES	$605.00	03/21/00

1. Change of correspondence address or indication of " Fee Address" (37 CFR 1.363). Use of PTO form(s) and Customer Number are recommended, but not required.

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47) attached.

2. For printing on the patent front page, list (1) the names of up to 3 registered patent attorneys or agents OR, alternatively, (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 BAKER BOTTS,

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)
PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. Inclusion of assignee data is only appropriate when an assignment has been previously submitted to the PTO or is being submitted under separate cover. Completion of this form is NOT a subsititue for filing an assignment.

(A) NAME OF ASSIGNEE ONCORMED, INC.

(B) RESIDENCE: (CITY & STATE OR COUNTRY) 205 Perry Parkway, Gaithersburg, MD 20877

Please check the appropriate assignee category indicated below (will not be printed on the patent)

☐ individual ☒ corporation or other private group entity ☐ government

4a. The following fees are enclosed (make check payable to Commissioner of Patents and Trademarks):

☒ Issue Fee

☐ Advance Order - # of Copies _____

4b. The following fees or deficiency in these fees should be charged to:

DEPOSIT ACCOUNT NUMBER 02-4377

(ENCLOSE AN EXTRA COPY OF THIS FORM)

☒ Issue Fee if insufficient

☐ Advance Order - # of Copies _____

The COMMISSIONER OF PATENTS AND TRADEMARKS IS requested to apply the Issue Fee to the application identified above.

(Authorized Signature) Janet M. MacLeod (Date) 3-6-2000

NOTE: The Issue Fee will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the Patent and Trademark Office.

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending on the needs of the individual case. Any comments on the amount of time required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND FEES AND THIS FORM TO: Box Issue Fee, Assistant Commissioner for Patents, Washington D.C. 20231

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

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TRANSMIT THIS FORM WITH FEE

CERTIFICATE

FEB 13 2001

OF CORRECTION

Corr #12

A31421 - 072880.0102
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Patentee	:	Lescallett e et al.			
Patent No.	:	6,051,379	Issued	:	April 18, 2000
Serial No.	:	08/984,034	Examiner	:	Lisa Arthur
Filed	:	December 2, 1997	Group Art Unit:		1643
For	:	CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2			

REQUEST FOR A CERTIFICATE OF CORRECTION
UNDER 37 C.F.R. § 1.322

I hereby certify that this paper is being deposited with the United States Postal Service as first class mail, in an envelope addressed to:
Hon. Commissioner for Patents and Trademarks, Washington, D.C. 20231

February 5, 2001
Date of Deposit

Janet M. MacLeod
Attorney Name

35,263
PTO Registration No.

Janet M MacLeod
Signature

February 5, 2001
Date of Signature

PUBLISH

Commissioner for Patents
ATTN: Certificate of Corrections Branch
Washington, D.C. 20231

Sir:

Upon comparison of U.S. Patent No. 6,051,379, granted April 18, 2000 to Lescallett et al. for CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2 with our file of the application therefor, several errors were found:

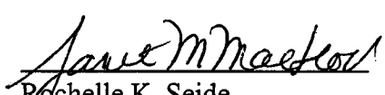
IN THE PATENT	IN THE APPLICATION FILE
In the Claims:	
Column 39, line 37: "according to claim 1" should be deleted;	per Amendment dated September 20, 1999, claim 14, line 1
Column 40, line 42: "6909" should read -- 6909, --;	per Examiner's Amendment dated December 21, 1999, claim 49, line 2
Column 40, line 45: "variations; and" should read -- variations in the BRCA2 gene; and --;	per Amendment dated January 7, 1999, claim 49, line 5
Column 40, line 47: "in the BRCA2 gene" (second occurrence) should be deleted;	per Claim 49, line 7
Column 40, line 50: "the" should be deleted;	per Examiner's Amendment dated December 21, 1999, claim 50, line 2
Column 41, line 54: "sequence variation" should read --sequence contained within the BRCA2 gene sequence or a polynucleotide having a sequence variation--;	per Amendment dated September 20, 1999, claim 62, lines 7–8
Column 41, line 61: "herein" should read -- wherein --;	per Amendment dated February 17, 1998, claim "66", line 1
Column 42, line 19: "containing:" should read -- containing an isolated nucleotide primer comprising the sequence: --;	per Amendments dated February 17, 1998 and September 20, 1999, claim "70"/67, line 4
Column 42, lines 56–57: "C2192G, 3772delTT, C5193G, 5374del4, 6495delGC, or 6909insG" should be deleted;	per Claim 75, line 1
Column 42, line 63: "while in" should read -- which is --;	per Amendment dated September 20, 1999, claim 75, line 1

REMARKS

A proposed Certificate of Correction is enclosed in duplicate. It is respectfully requested that the Certificate of Correction be issued for attachment to the original patent under the provisions of 37 C.F.R. §1.322.

Applicants do not believe any fee is required by this communication. However, the Commissioner is hereby authorized to charge any fee required by this communication to Deposit Account No. 02-4377.

Respectfully submitted,



Rochelle K. Seide
Patent Office Reg. No. 32,300

Janet M. MacLeod
PTO Reg. No. 35,263

Attorney for Applicants
(212) 408-2597

Enclosures

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,051,379

DATED : April 18, 2000

INVENTOR(S) : Lescallett et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims:

Column 39, line 37: "according to claim 1" should be deleted;

Column 40, line 42: "6909" should read -- 6909, --;

Column 40, line 45: "variations; and" should read -- variations in the BRCA2 gene; and --;

Column 40, line 47: "in the BRCA2 gene" (second occurrence) should be deleted;

Column 40, line 50: "the" should be deleted;

Column 41, line 54: "sequence variation" should read --sequence contained within the BRCA2 gene sequence or a polynucleotide having a sequence variation--;

Column 41, line 61: "herein" should read -- wherein --;

Column 42, line 19: "containing:" should read -- containing an isolated nucleotide primer comprising the sequence: --;

Column 42, lines 56–57: "C2192G, 3772delTT, C5193G, 5374del4, 6495delGC, or 6909insG" should be deleted;

Column 42, line 63: "while in" should read -- which is --;

MAILING ADDRESS OF SENDER:
Baker Botts L.L.P.
30 Rockefeller Plaza
New York, New York 10112

PATENT NO. 6,051,379

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UNITED STATES PATENT AND TRADEMARK OFFICE

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www.uspto.gov

PAYOR NUMBER
009629

P75M

MORGAN LEWIS & BOCKIUS LLP
1111 PENNSYLVANIA AVENUE NW
WASHINGTON DC 20004

DATE PRINTED
10/29/07

MAINTENANCE FEE REMINDER

According to the records of the U.S. Patent and Trademark Office (USPTO) the maintenance fee for the patent(s) listed below (for which the above address is on record as the fee address under 37 CFR 1.363) has not been paid within the six-month period set forth in 37 CFR 1.362(d). THE MAINTENANCE FEE MAY STILL BE PAID WITH THE APPLICABLE SURCHARGE SET FORTH IN 37 CFR 1.20(h), WITHIN THE SIX-MONTH GRACE PERIOD SET FORTH IN 37 CFR 1.362(e).

Unless payment of the maintenance fee and the applicable surcharge is received in the USPTO within the six-month grace period, THE PATENT WILL EXPIRE AS OF THE END OF THE GRACE PERIOD. 35 U.S.C. 41(b).

The total payment due is the amount required on the date the fee is paid (and not necessarily the amount indicated below). All USPTO fees (including maintenance fees) are subject to change. Customers should refer to the USPTO Web site (www.uspto.gov) or call the Maintenance Fee Branch at 571-272-6500 for the most current fee amounts for the correct entity status before submitting payment. The total payment due indicated below is based on the entity status according to current Office records (shown below).

Timely payment of the total payment due is required in order to avoid expiration of the patent. A maintenance fee payment can be timely made using the certificate of mailing or transmission procedure set forth in 37 CFR 1.8.

PATENT NUMBER	FEE AMT	MAINT. SURCHG	U.S. APPL NUMBER	PATENT ISSUE DATE	APPL. FILING DATE	PAY-MENT YEAR	SMALL ENTITY?	TOTAL PYMT DUE	ATTORNEY DOCKET NUMBER
[redacted]60	130		08984034	04/18/00	12/02/97	8	NO	2490	A314202880

The maintenance fee and the applicable surcharge can be paid quickly and easily over the Internet at www.uspto.gov by electronic funds transfer (EFT), credit card, or USPTO deposit account payment methods. The mailing address for all maintenance fee payments not electronically submitted over the Internet is: U.S. Patent and Trademark Office, P.O. Box 979070, St. Louis, MO 63197-9000.

Direct any questions about this notice to: Mail Stop M Correspondence, Director of the United States Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450.

NOTE: This notice was automatically generated based on the amount of time that elapsed since the date a patent was granted. It is possible that the patent term may have ended or been shortened due to a terminal disclaimer that was filed in the application. Also, for any patent that issued from an application filed on or after June 8, 1995 containing a specific reference to an earlier filed application or applications under 35 U.S.C. 120, 121, or 365(c), the patent term ends 20 years from the date on which the earliest such application was filed, unless the term was adjusted or extended under 35 U.S.C. 154 or 156. Patentee should determine the relevant patent term for a patent before paying the maintenance fee.

MF4401 (7/2007)

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

(FOR USE WITH FORM PTO-875)

SERIAL NO.	FILING DATE
APPLICANT(S)	

CLAIMS

	AS FILED		AFTER 1st AMENDMENT		AFTER 2nd AMENDMENT	
	IND.	DEP.	IND.	DEP.	IND.	DEP.
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TOTAL CLAIMS						

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TOTAL DEP.						
TOTAL CLAIMS						

* *MAY BE USED FOR ADDITIONAL CLAIMS OR ADMENDMENTS*

FORM **PTO-1360** (REV. 3-78)

U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office

Claims are misnumbered

PATENT APPLICATION FEE DETERMINATION RECORD

Effective October 1, 1997

Application or Docket Number: 08984084

CLAIMS AS FILED - PART I

	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY TYPE [2] RATE	SMALL ENTITY FEE	OR	OTHER THAN SMALL ENTITY RATE	OTHER THAN SMALL ENTITY FEE
FOR							
BASIC FEE				395.00	OR		790.00
TOTAL CLAIMS	75 minus 20 =	* 55	x$11=	605	OR	x$22=	1210
INDEPENDENT CLAIMS	8 minus 3 =	* 5	x41=	205	OR	x82=	410
MULTIPLE DEPENDENT CLAIM PRESENT			+135=		OR	+270=	
			TOTAL	1205	OR	TOTAL	2410

* If the difference in column 1 is less than zero, enter "0" in column 2

CLAIMS AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				

SMALL ENTITY RATE	SMALL ENTITY ADDITIONAL FEE	OR	OTHER THAN SMALL ENTITY RATE	OTHER THAN SMALL ENTITY ADDITIONAL FEE
x$11=		OR	x$22=	
x41=		OR	x82=	
+135=		OR	+270=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				

SMALL ENTITY RATE	SMALL ENTITY ADDITIONAL FEE	OR	OTHER THAN SMALL ENTITY RATE	OTHER THAN SMALL ENTITY ADDITIONAL FEE
x$11=		OR	x$22=	
x41=		OR	x82=	
+135=		OR	+270=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT C

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
Total	*	Minus	**	=
Independent	*	Minus	***	=
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				

SMALL ENTITY RATE	SMALL ENTITY ADDITIONAL FEE	OR	OTHER THAN SMALL ENTITY RATE	OTHER THAN SMALL ENTITY ADDITIONAL FEE
x$11=		OR	x$22=	
x41=		OR	x82=	
+135=		OR	+270=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3."
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

US006051379A

United States Patent [19]

Lescallett et al.

[11] **Patent Number: 6,051,379**

[45] **Date of Patent: *Apr. 18, 2000**

[54] **CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2**

[75] Inventors: **Jennifer Lee Lescallett**, Great Falls, Va.; **Tammy Lawrence**, Laurel; **Antonette Preisinger Allen**, Severn, both of Md.; **Sheri Jon Olson**, Falls Church, Va.; **Denise Bernadette Thurber**, Silver Spring; **Marga Belle White**, Frederick, both of Md.

[73] Assignee: **Oncormed, Inc.**, Gaithersburg, Md.

[*] Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

[21] Appl. No.: **08/984,034**

[22] Filed: **Dec. 2, 1997**

Related U.S. Application Data

[60] Provisional application No. 60/059,595, Sep. 23, 1997.

[51] **Int. Cl.**[7] **C12Q 1/68**; C07H 21/04

[52] **U.S. Cl.** **435/6**; 536/24.31; 536/24.33

[58] **Field of Search** 536/24.33, 24.31, 536/24.5; 435/6, 91.2, 320.1

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,683,195 7/1987 Mullis et al. 435/6

OTHER PUBLICATIONS

Panayiotiais P., et al. British Journal of Haematology 97:844–847 (1997).

Tavitigian et al. Nature Genetics 12: 333–337 (1996).

Primary Examiner—Lisa B. Arthur
Attorney, Agent, or Firm—Baker Botts

[57] **ABSTRACT**

New mutations have been found in the BRCA2 gene. The mutations are located at nucleotide numbers 2192, 3772, 5193, 5374, 6495 or 6909 of the published nucleotide sequence of BRCA2 gene. A process for identifying a sequence variation in a BRCA2 polynucleotide sequence is disclosed. The identification process includes allele specific sequence-based assays of known sequence variations. The methods can be used for efficient, and accurate detection of a mutation in a test BRCA2 gene sample.

44 Claims, No Drawings

CANCER SUSCEPTIBILITY MUTATIONS OF BRCA2

This application is in part based on provisional patent application 60/059,595 filed Sep. 23, 1997, the contents are incorporated by reference.

FIELD OF THE INVENTION

This invention relates to the breast cancer succeptibility gene BRCA2. More specifically, this invention detects germline mutations of the BRCA2 gene that are associated with a predisposition to breast, ovarian and associated cancers. Methods and reagents for detecting the presence of these mutations are included.

BACKGROUND OF THE INVENTION

BRCA2, located on chromosome 13q12-q13, consists of over 70 kb of genomic DNA. The coding sequence produces a protein of 3,418 amino acids. Although most of the exons are small, exons 10 and 11 represent approximately 60% of the entire coding region. BRCA2 is thought to be a tumor suppressor gene associated with breast and ovarian cancer. Thus mutantions which form an altered tumor suppressor or altered concentrations of tumor suppressor may be indicative of a higher succeptibility to certain cancers.

The nucleotide sequence for at least one BRCA2 gene is known and is reported in GENBANK accession Number U43746. The BRCA2 gene sequence is available on the Breast Cancer Information Core.

Germline mutations of BRCA2 are predicted to account for approximately 35% of families with multiple case, early onset female breast cancer, and they are also associated with an increased risk of male breast cancer, ovarian cancer, prostrate cancer and pancreatic cancer.

The location of one or more mutations of the BRCA2 gene provides a promising approach to reducing the high incidence and mortality associated with breast and ovarian cancer through the early detection of women at high risk. These women, once identified, can be targeted for more aggressive prevention programs. Screening is carried out by a variety of methods which include karyotyping, probe binding and DNA sequencing. In such cases where one or only a few known mutations are responsible for the disease, such as testing family members, methods for detecting the mutations are targeted to the site within the gene at which they are known to occur.

Many mutations and normal polymorphisms have already been reported in the BRCA2 gene. A world wide web site has been built to facilitate the detection and characterization of alterations in breast cancer susceptibility genes. Such mutations in BRCA2 can be accessed through the Breast Cancer Information Core at:
HTTP://www.nchgr.nih.gov/dir/lab_transfer/bic.

While mutations occur throughtout the BRCA2 gene, there is a need for a high sample number (throughput), sensitivity, accuracy and cost effectiveness. Identification of mutations of the BRCA2 gene would allow more widespread diagnostic screening for hereditary breast and ovarian cancer than is currently possible and permit identification of functional areas deduced from the mutational spectrum observed.

SUMMARY OF THE INVENTION

The present invention is based on the discovery of six mutations in the BRCA2 gene sequence which is associated with susceptibility to and development of breast and ovarian cancer. Specifically, mutations located at nucleotide numbers 2192, 3772, 5193, 5374, 6495 and 6909 have been discovered.

It is an object of the invention to provide a method for determining a predisposition or higher susceptibility to breast, ovarian and other cancers.

It is another object of the invention to provide primers for detecting and amplifying a region of DNA which contains the BRAC2 mutations.

It is another object of the invention to provide probes for detecting a region of DNA which contains the BRAC2 mutations.

It is a further object of the invention to provide a method of characterizing and classifying a tumor and determining a therapy dependant upon the type of mutation(s) present.

It is also an object of the present invention to provide a mutant BRCA2 gene and expressed mutant protein for drug development, gene therapy and other uses to prevent or amelorate the effects of or resulting from the mutant BRCA2 gene.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

For defining the present invention, the following nomenclature is used to describe the mutation due to an inconsistency in the published literature. Beaudet et al, *Human Mutations,* 2: 245–248 (1993), Antonarakis et al, *Human Mutations,* 4: 166 (1994), Cotton, *Human Mutations,* 8: 197–202 (1996), and Beutler et al, *Human Mutations,* 8: 203–206 (1996). In defining the mutation, the number indicates the nucleotide number corresponding to the BRCA2 gene sequence where the mutation first occurs. Other BRCA2 sequences (haplotypes) which are polymorphisms or genetic variations of BRCA2 may used, in which a corresponding mutation at the corresponding nucleotide number are present. Different sequence variations in a normal BRCA1 gene have been discovered previously by the inventors (U.S. Pat. No. 5,654,155) and sequence variations in a normal BRCA2 gene sequence are expected. Also note Shattuck-Eidens, et al, *Journal of the American Medical Association,* 278: p. 1242 (1997). Generally, the sense strand is referred to. For simplified identification purposes of this application, reference is to the BRCA2 sequence referenced above, however the invention is equally applicable to all of the normal BRCA2 sequences.

Insertion mutations are indicated by "ins" and deletion mutations are indicated by "del". The letters after "ins" or "del" refer to the nucleotide(s) which were inserted or deleted. Insertions and deletions above two nucleotides are indicated by the number of nucleotides inserted or deleted. When the mutation results in one nucleotide being substituted for another, the nucleotide of the BRCA2 gene sequence is placed to the left of the number and the nucleotide found in the mutation is placed to the right of the number.

The first mutation is referred to as C2192G. This mutation or genetic alteration causes a change in nucleotide number 2192 from C to G resulting in codon 655 being changed from proline to arginine. Any amino acid change can have a dramatic change in biological activity. Some people believe that since proline can form a turn in the chain of amino acids in the protein, the removal of this turn, particularly when substituted with a charged amino acid may change the three dimentional configuration of the protein or at least may negatively affect on the biological activity of the resulting protein.

The second mutation is referred to as 3772delTT. This mutation deletes TT at nucleotide number 3772 causing a frameshift mutation and forming an in-frame stop codon at codon 1182. It has been demonstrated that a truncated, and most likely non-functional, protein has been produced by this mutation.

The third mutation is referred to as C5193G. This mutation substitutes G for C at nucleotide number 5193 causing a stop codon (TAG) to be formed at codon 1655. It has been demonstrated that a truncated, and most likely non-functional, protein has been produced by this mutation.

The fourth mutation is referred to as 5374del4. This mutation deletes TATG at nucleotide number 5374 causing a frameshift mutation and forming an in-frame stop at codon 1723. It has been demonstrated that a truncated, and most likely non-functional, protein has been produced by this mutation.

The fifth mutation is referred to as 6495delGC. This mutation deletes GC at nucleotide number 6495 causing a frameshift mutation and forming an in-frame stop codon at codon 2090. It has been demonstrated that a truncated, and most likely non-functional, protein has been produced by this mutation.

The sixth mutation is referred to as 6909insG. This mutation inserts a G at nucleotide number 6909 causing a frameshift mutation and forming an in-frame stop codon at codon 2232. It has been demonstrated that a truncated, and most likely non-functional, protein has been produced by this mutation.

The presence of truncated proteins was demonstrated by expression of overlapping portions of the mutant genes and measuring molecular weight by gel electrophoresis.

Useful DNA molecules according to the present invention are those which will specifically hybridize to BRCA2 sequences in the region of the C2192G, 3772delTT, C5193G, 5374del4, 6495delGC or 6909insG mutations. Typically these DNA molecules are 17 to 20 nucleotides in length (longer for large insertions) and have the nucleotide sequence corresponding to the region of the mutations at their respective nucleotide locations on the BRCA2 gene sequence. Such molecules can be labeled, according to any technique known in the art, such as with radiolabels, fluorescent labels, enzymatic labels, sequence tags, biotin, other ligands, etc.

According to another aspect of the invention, the DNA molecules, or oligonucleotides, contain one or more of the specific mutations. Generally it is preferred for each DNA probe to encompass only one mutation. Such molecules may be labeled and can be used as allele-specific oligonucleotide probes to detect the mutation of interest.

Polynucleotide containing biological samples, such as blood, can be tested to determine whether the BRCA2 gene contains one of the specific mutations listed above. To amplify the BRCA2 gene, one may use polymerase chain reaction (PCR) using primers which hybridize to the ends of the exons or to the introns flanking the exons. In the situation of exon 11, the exon is so large that using plural pairs of primers to amplify overlapping regions is preferred. Such was actually used in the Examples below.

Amplification may also be performed by a number of other techniques such as by cloning the gene and linking the BRCA2 gene or fragments thereof in the sample to a vector. "Shot gun" cloning is particularly preferred. For the purposes of this application, a vector may be any polynucleotide containing system which induces replication such as a plasmid, cosmid, virus, transposon, or portions thereof.

In one embodiment of the invention a pair of isolated oligonucleotide primers are provided.

BRCA2-11F 5'TGG TAC TTT AAT TTT GTC ACT T3' SEQ ID NO:1

BRCA2-11R 5'TGC AGG CAT GAC AGA GAA T3' SEQ ID NO:2

The designation BRCA2-11 refers to a sequence in or near exon 11 of the BRCA2 gene. F and R refer to forward and reverse.

The oligonucleotide primers are useful in directing amplification of a target polynucleotide prior to sequencing. These unique BRCA2 exon 11 oligonucleotide primers were used to scan the BRCA2 gene to find the mutations. From the sequence information, the probes were designed and produced to assay for the mutation based upon identification of the C2192G mutation.

In another embodiment of the invention a pair of isolated allele specific oligonucleotide probes are provided.

5'TGA AGA ACC AAC TTT GT3' SEQ ID NO:3

5'TGA AGA ACG AAC TTT GT3' SEQ ID NO:4

These allele specific oligonucleotides are useful in diagnosis of a subject at risk of having breast or ovarian cancer. The allele specific oligonucleotides hybridize with a target polynucleotide sequence containing the C2192G mutation. 5'TGA AGA ACC AAC TTT GT3', SEQ ID NO:3, hybridizes preferentially to the wildtype sequence and is useful as a control sequence. 5'TGA AGA ACG AAC TTT GT3', SEQ ID NO:4, is designed to hybridize preferentially to the mutant sequence.

In a second embodiment of the invention a pair of isolated oligonucleotide primers are provided.

BRCA2-11F 5'CTC AGA TGT TAT TTT CCA AGC3' SEQ ID NO:5

BRCA2-11R 5'CTG TTA AAT AAC CAG AAG CAC3' SEQ ID NO:6

The oligonucleotide primers are useful in directing amplification of a target polynucleotide prior to sequencing. These unique BRCA2 exon 11 oligonucleotide primers were used to scan the BRCA2 gene to find the mutations. From the sequence information, the probes were designed and produced to assay for the mutation based upon identification of the 3772delTT mutation.

In another embodiment of the invention a pair of isolated allele specific oligonucleotides are provided.

5'GCA AGC AAT TTG AAG GT3' SEQ ID NO:7

5'GCA AGC AAT GAA GGT AC3' SEQ ID NO:8

These allele specific oligonucleotides are useful in diagnosis of a subject at risk of having breast or ovarian cancer. The allele specific oligonucleotides hybridize with a target polynucleotide sequence containing the 3772delTT mutation. 5'GCA AGC AAT TTG AAG GT3', SEQ ID NO:7, hybridizes preferentially to the wildtype sequence and is useful as a control sequence. 5'GCA AGC AAT GAA GGT AC3', SEQ ID NO:8, is designed to hybridize preferentially to the mutant sequence.

In a third embodiment of the invention a pair of isolated oligonucleotide primers are provided.

BRCA2-11F 5'GCA AAG ACC CTA AAG TAC AG3', SEQ ID NO:9

BRCA2-11R 5'CAT CAA ATA TTC CTT CTC TAA G3', SEQ ID NO:10

The oligonucleotide primers are useful in directing amplification of a target polynucleotide prior to sequencing. These unique BRCA2 exon 11 oligonucleotide primers were used to scan the BRCA2 gene to find the mutations. From the sequence information, the probes were designed and produced to assay for the mutation based upon identification of the C5193G mutation.

In another embodiment of the invention a pair of isolated allele specific oligonucleotides are provided.

5'ACT TGT TAC ACA AAT CA3', SEQ ID NO:11

5'ACT TGT TAG ACA AAT CA3', SEQ ID NO:12

These allele specific oligonucleotides are useful in diagnosis of a subject at risk of having breast or ovarian cancer. The allele specific oligonucleotides hybridize with a target polynucleotide sequence containing the C5193G mutation. 5'ACT TGT TAC ACA AAT CA3', SEQ ID NO:11, hybridizes preferentially to the wildtype sequence and is useful as

a control sequence. 5'ACT TGT TAG ACA AAT CA3', SEQ ID NO:12, is designed to hybridize preferentially to the mutant sequence.

In a fourth embodiment of the invention a pair of isolated oligonucleotide primers are provided.

BRCA2-11F 5'GAA AAT TCA GCC TTA GC3' SEQ ID NO:13

BRCA2-11R 5'ATC AGA ATG GTA GGA AT3' SEQ ID NO:14

The oligonucleotide primers are useful in directing amplification of a target polynucleotide prior to sequencing. These unique BRCA2 exon 11 oligonucleotide primers were used to scan the BRCA2 gene to find the mutations. From the sequence information, the probes were designed and produced to assay for the mutation based upon identification of the 5374del4 mutation.

In another embodiment of the invention a pair of isolated allele specific oligonucleotides are provided.

5'ATT ATT TGT ATG AAA AT3' SEQ ID NO:15

5'ATT ATT TGA AAA TAA TT3' SEQ ID NO:16

These allele specific oligonucleotides are useful in diagnosis of a subject at risk of having breast or ovarian cancer. The allele specific oligonucleotides hybridize with a target polynucleotide sequence containing the 5374del4 mutation. 5'ATT ATT TGT ATG AAA AT3', SEQ ID NO:15, hybridizes preferentially to the wildtype sequence and is useful as a control sequence. 5'ATT ATT TGA AAA TAA TT3', SEQ ID NO:16, is designed to hybridize preferentially to the mutant sequence.

In a fifth embodiment of the invention a pair of isolated oligonucleotide primers are provided.

BRCA2-11F 5'TAC AGC AAG TGG AAA GC3' SEQ ID NO:17

BRCA2-11R 5'AAG TTT CAG TTT TAC CAA T3' SEQ ID NO:18

The oligonucleotide primers are useful in directing amplification of a target polynucleotide prior to sequencing. These unique BRCA2 exon 11 oligonucleotide primers were used to scan the BRCA2 gene to find the mutations. From the sequence information, the probes were designed and produced to assay for the mutation based upon identification of the 6495delGC mutation.

In another embodiment of the invention a pair of isolated allele specific oligonucleotides are provided.

5'GAA CTG AGC ATA GTC TT3' SEQ ID NO:19

5'GAA CTG AAT AGT CTT CA3' SEQ ID NO:20

These allele specific oligonucleotides are useful in diagnosis of a subject at risk of having breast or ovarian cancer. The allele specific oligonucleotides hybridize with a target polynucleotide sequence containing the 6495delGC mutation. 5'GAA CTG AGC ATA GTC TT3', SEQ ID NO:19, hybridizes preferentially to the wildtype sequence and is useful as a control sequence. 5'GAA CTG AAT AGT CTT CA3', SEQ ID NO:20, is designed to hybridize preferentially to the mutant sequence.

In a sixth embodiment of the invention a pair of isolated oligonucleotide primers are provided.

BRCA2-11F 5'ACT TTT TCT GAT GTT CCT GTG3' SEQ ID NO:21

BRCA2-11R 5'TAA AAA TAG TGA TTG GCA ACA3' SEQ ID NO:22

The oligonucleotide primers are useful in directing amplification of a target polynucleotide prior to sequencing. These unique BRCA2 exon 11 oligonucleotide primers were used to scan the BRCA2 gene to find the mutations. From the sequence information, the probes were designed and produced to assay for the mutation based upon identification of the 6909insG mutation.

In another embodiment of the invention a pair of isolated allele specific oligonucleotides are provided.

5'CAG AAG CAG TAG AAA TT3' SEQ ID NO:23

5'CAG AAG CAG GTA GAA AT3' SEQ ID NO:24

These allele specific oligonucleotides are useful in diagnosis of a subject at risk of having breast or ovarian cancer. The allele specific oligonucleotides hybridize with a target polynucleotide sequence containing the 6909insG mutation. 5'CAG AAG CAG TAG AAA TT3', SEQ ID NO:23, hybridizes preferentially to the wildtype sequence and is useful as a control sequence. 5'CAG AAG CAG GTA GAA AT3', SEQ ID NO:24, is designed to hybridize preferentially to the mutant sequence.

The primers of the invention embrace oligonucleotides of sufficient length and appropriate sequence to provide initiation of polymerization on a significant number of nucleic acids in the polymorphic locus.

Preferred sequences for the present invention are SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:21, and SEQ ID NO:22. Environmental conditions conducive to synthesis of extension products include the presence of nucleoside triphosphates, an agent for polymerization, such as DNA polymerase, and suitable conditions such as temperature, ionic strength and pH. The primer is preferably single stranded for maximum efficiency in amplification, but may be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent for polymerization. The exact length of primer will depend on many factors, including temperature, buffer, and nucleotide composition. The oligonucleotide primer typically contains 12–20 or more nucleotides, although it may contain fewer nucleotides.

Primers of the invention are designed to be "substantially" complementary to each strand of the genomic locus to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands under conditions which allow the agent for polymerization to perform. In other words, the primers should have sufficient complementarity with the 5' and 3' sequences flanking the mutation to hybridize therewith and permit amplification of the genomic locus.

Oligonucleotide primers of the invention are employed in the amplification process which is an enzymatic chain reaction that produces exponential quantities of polymorphic locus relative to the number of reaction steps involved. Typically, one primer is complementary to the negative (−) strand of the polymorphic locus and the other is complementary to the positive (+) strand. Annealing the primers to denatured nucleic acid followed by extension with an enzyme, such as the large fragment of DNA polymerase I (Klenow) and nucleotides, results in newly synthesized + and − strands containing the target polymorphic locus sequence. Because these newly synthesized sequences are also templates, repeated cycles of denaturing, primer annealing, and extension results in exponential production of the region (i.e., the target polymorphic locus sequence) defined by the primers. The product of the chain reaction is a discreet nucleic acid duplex with termini corresponding to the ends of the specific primers employed.

The oligonucleotide primers of the invention may be prepared using any suitable method, such as conventional phosphotriester and phosphodiester methods or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage, et al., *Tetrahedron Letters,* 22:1859–1862, (1981). One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066.

Any nucleic acid specimen, in purified or nonpurified form, can be utilized as the starting nucleic acid or acids,

providing it contains, or is suspected of containing, the specific nucleic acid sequence containing the polymorphic locus. Thus, the process may amplify, for example, DNA or RNA, including messenger RNA, wherein DNA or RNA may be single stranded or double stranded. In the event that RNA is to be used as a template, enzymes, and/or conditions optimal for reverse transcribing the template to DNA would be utilized. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of nucleic acids may also be employed, or the nucleic acids produced in a previous amplification reaction herein, using the same or different primers may be so utilized. The specific nucleic acid sequence to be amplified, i.e., the polymorphic locus, may be a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor fraction of a complex mixture, such as contained in whole human DNA.

DNA utilized herein may be extracted from a body sample, such as blood, tissue material and the like by a variety of techniques such as that described by Maniatis, et. al. in *Molecular Cloning:A Laboratory Manual*, Cold Spring Harbor, N.Y., p 280–281, 1982). If the extracted sample is impure, it may be treated before amplification with an amount of a reagent effective to open the cells, or animal cell membranes of the sample, and to expose and/or separate the strand(s) of the nucleic acid(s). This lysing and nucleic acid denaturing step to expose and separate the strands will allow amplification to occur much more readily.

The deoxyribonucleotide triphosphates dATP, dCTP, dGTP, and dTTP are added to the synthesis mixture, either separately or together with the primers, in adequate amounts and the resulting solution is heated to about 90°–100° C. from about 1 to 10 minutes, preferably from 1 to 4 minutes. This is sufficient to denature any double strands. After this heating period, the solution is allowed to cool at a rate which is preferable for the primer hybridization. To the cooled mixture is added an appropriate agent for effecting the primer extension reaction (called herein "agent for polymerization"), and the reaction is allowed to occur under conditions known in the art. The agent for polymerization may also be added together with the other reagents if it is heat stable. This synthesis (or amplification) reaction may occur at room temperature up to a temperature above which the agent for polymerization no longer functions. Thus, for example, if DNA polymerase is used as the agent, the temperature is generally no greater than about 40° C. Thermostable DNA polymerases, such as Taq polymerase may function at a higher temperature.

The agent for polymerization may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase, polymerase muteins, reverse transcriptase, other enzymes, including heat-stable enzymes (i.e., those enzymes which perform primer extension after being subjected to temperatures sufficiently elevated to cause denaturation), such as Taq polymerase. The suitable enzyme will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each polymorphic locus nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths.

The newly synthesized strand and its complementary nucleic acid strand will form a double-stranded molecule under hybridizing conditions described above and this hybrid is used in subsequent steps of the process. In the next step, the newly synthesized double-stranded molecule is subjected to denaturing conditions using any of the procedures described above to provide single-stranded molecules.

The steps of denaturing, annealing, and extension product synthesis can be repeated as often as needed to amplify the target polymorphic locus nucleic acid sequence to the extent necessary for detection. The amount of the specific nucleic acid sequence produced will accumulate in an exponential fashion. PCR. A Practical Approach, ILR Press, Eds. M. J. McPherson, P. Quirke, and G. R. Taylor, 1992.

The amplification products may be detected by analyzing it by Southern blots without using radioactive probes. In such a process, for example, a small sample of DNA containing a very low level of the nucleic acid sequence of the polymorphic locus is amplified, and analyzed via a Southern blotting technique or similarly, using dot blot analysis. The use of non-radioactive probes or labels is facilitated by the high level of the amplified signal. Alternatively, probes used to detect the amplified products can be directly or indirectly detectably labeled, for example, with a radioisotope, a fluorescent compound, a bioluminescent compound, a chemiluminescent compound, a metal chelator or an enzyme. Those of ordinary skill in the art will know of other suitable labels for binding to the probe, or will be able to ascertain such, using routine experimentation. In the preferred embodiment, the amplification products are determinable by separating the mixture on an agarose gel containing ethidium bromide which causes DNA to be fluorescent.

Sequences amplified by the methods of the invention can be further evaluated, detected, cloned, sequenced, and the like, either in solution or after binding to a solid support, by any method usually applied to the detection of a specific DNA sequence such as PCR, oligomer restriction (Saiki, et.al., *Bio/Technology,* 3:1008–1012, 1985), allele-specific oligonucleotide (ASO) probe analysis (Conner, et. al., *Proc. Natl. Acad. Sci. U.S.A.,* 80:278, 1983), oligonucleotide ligation assays (OLAs) (Landgren, et. al., *Science,* 241:1007, 1988), and the like. Molecular techniques for DNA analysis have been reviewed (Landgren, et. al., *Science,* 242:229–237, 1988).

Preferably, the method of amplifying is by PCR, as described herein and as is commonly used by those of ordinary skill in the art. Alternative methods of amplification have been described and can also be employed as long as the BRCA2 locus amplified by PCR using primers of the invention is similarly amplified by the alternative means. Such alternative amplification systems include but are not limited to self-sustained sequence replication, which begins with a short sequence of RNA of interest and a T7 promoter. Reverse transcriptase copies the RNA into cDNA and degrades the RNA, followed by reverse transcriptase polymerizing a second strand of DNA. Another nucleic acid amplification technique is nucleic acid sequence-based amplification (NASBA) which uses reverse transcription and T7 RNA polymerase and incorporates two primers to target its cycling scheme. NASBA can begin with either DNA or RNA and finish with either, and amplifies to 10^8 copies within 60 to 90 minutes. Alternatively, nucleic acid can be amplified by ligation activated transcription (LAT). LAT works from a single-stranded template with a single primer that is partially single-stranded and partially double-stranded. Amplification is initiated by ligating a cDNA to the promoter oligonucleotide and within a few hours, amplification is 10^8 to 10^9 fold. The QB replicase system can be utilized by attaching an RNA sequence called MDV-1 to RNA complementary to a DNA sequence of interest. Upon mixing with a sample, the hybrid RNA finds its complement among the specimen's mRNAs and binds, activating the replicase to copy the tag-along sequence of interest. Another nucleic acid amplification technique, ligase chain reaction

(LCR), works by using two differently labeled halves of a sequence of interest which are covalently bonded by ligase in the presence of the contiguous sequence in a sample, forming a new target. The repair chain reaction (RCR) nucleic acid amplification technique uses two complementary and target-specific oligonucleotide probe pairs, thermostable polymerase and ligase, and DNA nucleotides to geometrically amplify targeted sequences. A 2-base gap separates the oligonucleotide probe pairs, and the RCR fills and joins the gap, mimicking normal DNA repair. Nucleic acid amplification by strand displacement activation (SDA) utilizes a short primer containing a recognition site for Hinc II with short overhang on the 5' end which binds to target DNA. A DNA polymerase fills in the part of the primer opposite the overhang with sulfur-containing adenine analogs. Hinc II is added but only cuts the unmodified DNA strand. A DNA polymerase that lacks 5' exonuclease activity enters at the cite of the nick and begins to polymerize, displacing the initial primer strand downstream and building a new one which serves as more primer. SDA produces greater than 10^7-fold amplification in 2 hours at 37° C. Unlike PCR and LCR, SDA does not require instrumented Temperature cycling. Another amplification system useful in the method of the invention is the QB Replicase System. Although PCR is the preferred method of amplification in the invention, these other methods can also be used to amplify the BRCA2 locus as described in the method of the invention.

In another embodiment of the invention, a method is provided for diagnosing a subject having a predisposition or higher susceptibility to (at risk of) breast or ovarian cancer comprising sequencing a target nucleic acid of a sample from a subject by dideoxy sequencing following amplification of the target nucleic acid. In such an embodiment, one does not even need to use any of the oligonucleotides, either primers or probes, described herein. The BRCA2 gene, or fragments thereof, may be directly cloned and then sequenced (such as by dideoxy methods) to determine the presence or absence of a mutation. In such a situation, one need only compare the sequence obtained to a naturally occurring (wild type) BRCA2 gene, or portion thereof.

Other methods of DNA sequencing such as those of Sanger et al, *Proc. Natl. Acad. Sci. USA,* 74: 5463 (1977) or Maxam et al, *Proc. Natl. Acad. Sci. USA,* 74: 560 (1977) or other methods known in the art may be used.

In another embodiment of the invention a method is provided for diagnosing a subject having a predisposition or higher susceptibility to (at risk of) breast or ovarian cancer comprising contacting a target nucleic acid of a sample from a subject with a reagent that detects the presence of one of the mutations of the present invention and detecting the mutation.

In another embodiment of the invention, a method and reagents are provided for repairing the gene mutation in at least some cells by applying an oligomer comprising the sequence of the wild-type probes to repair the individual's genome by triple strand hybridization. See U.S. Pat. Nos. 5,650,316 and 5,624,803 for example. This is a form of gene therapy to correct the defect in either apparently normal tissue or in an active tumor. Gene repair may also be performed on excized tumor cells which may be helpful in determining the preferred therapy to be used, particularly the reagents used for gene therapy. Other forms of gene therapy, such as providing a complete copy of a normal BRCA2 gene may also be used.

In another embodiment of the invention a method is provided for characterizing a tumor. Histologic type, morphologic grade, differences between inherited and sporadic breast cancer do not appear to be distinguished. One method comprises sequencing the target nucleic acid isolated from the tumor or other biological sample to determine if the mutation is has occured or is present. Sanger, F., et al., *J. Mol. Biol.,* 142:1617 (1980).

Characterizing a tumor as having originated from an inherited breast cancer gene may be clinically significant as the prevalence of bilateral breast cancer is higher than in sproadic cases. Weber, *Scientific American,* January–February p. 12–21 (1996). The tumor may be classified based on tissue taken from the tumor itself or from a non-tumor site which contains genomic DNA.

Yet another embodiment of the present invention is an isolated mutant BRCA2 DNA sequence which may be the entire sequence, an exon thereof or a fragment thereof. The DNA sequence must contain at least one mutation from the list: C2192G, 3772delTT, C5193G, 5374del4, 6495delGC or 6909insG. Preferably, the isolated DNA sequence contains a sequence complementary to at least one of the following: SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:12:, SEQ ID NO:16, SEQ ID NO:20, or SEQ ID NO:24. This sequence has usefulness alone, or after cloning and expression to determine suitable treatments to prevent formation of a tumor, prevent transmission of the mutant gene to offspring or to decide other prophylactic diagnostic and treatment protocols. The isolated DNA sequence may also be used for drug design by protein replacement, protein mimetics, screening known and unknown compounds, anti-idiotype antibodies to the BRCA1 active site for the preparation of an immunogen or vaccine and determining appropriate gene therapy to counter the pathology associated with the mutant BRCA2 gene. For diagnostic purposes, knowing the mutant BRCA2 sequence for comparison purposes is the critical step in diagnosis.

Another method comprises contacting a target nucleic acid of a sample from a subject with a reagent that detects the presence of the mutation and detecting the mutation. A number of hybridization methods are well known to those skilled in the art. Many of them are useful in carrying out the invention.

The materials for use in the method of the invention are ideally suited for the preparation of a diagnostic kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, tubes, and the like, each of the container means comprising one or more of the separate elements to be used in the method. For example, one of the container means may comprise means for amplifying BRCA2 DNA, said means comprising the necessary enzyme(s) and oligonucleotide primers for amplifying said target DNA from the subject. Another container may contain oligonucleotide probes for detecting the presence or absence of a mutation.

The oligonucleotide primers include primers having a sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:21, SEQ ID NO:22 or primer sequences substantially complementary or substantially homologous thereto. Other primers flanking the BRCA2 locus or a region containing one of the mutation sites may be used. The target flanking 5' and 3' polynucleotide sequence include other oligonucleotide primers for amplifying the BRCA2 locus will be known or readily ascertainable to those of skill in the art.

Oligonucleotide probes including probes having substantially the sequence of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:23, SEQ ID NO:24. Other oligonucleotide probes which hybridize to one or more of the BRCA2 mutation sites and sequences substantially complementary or homologous thereto may be used. Other oligonucleotide probes for detecting the mutations will be known or readily ascertainable to those of skill in the art.

The following definitions are provided for the purpose of understanding this invention.

The term "primer" as used herein refers to a sequence comprising two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and more preferably more than eight and most preferably at least 20 nucleotides of the BRCA2 gene wherein the sequence corresponds to a sequence flanking one of the mutations or wild type sequences of BRCA2 corresponding to the mutation sites. Primers may be used to initiate DNA synthesis via the PCR. The primers of the present invention include the sequences recited and complementary sequences which would aneal to the opposite DNA strand of the sample target. Since both strands of DNA are complementary and mirror images of each other, the same segment of DNA will be amplified.

The term "substantially complementary to" or "substantially the sequence" refers to sequences which hybridize to the sequences provided under stringent conditions and/or sequences having sufficient homology with, (e.g. SEQ ID NO:3 and SEQ ID NO:4) such that the allele specific oligonucleotides of the invention hybridize to the sequence. "Substantially" the same as it refers to oligonucleotide sequences also refers to the functional ability to hybridize or aneal with sufficient specificity to distinguish between the presence or absence of the mutation. This is measurable by the temperature of melting being sufficiently different to permit easy identification of whether the oligonucleotide is binding to the normal or mutant BRCA2 gene sequence.

The term "isolated" as used herein refers to being substantially free of other polynucleic acids, proteins, lipids, carbohydrates or other materials with which they may be associated. Such association being either in cellular material or in a synthesis medium.

"Biological sample" refers to a polynucleotide containing sample originally from a biological source. The sample may be from a living, dead or even archeological source from a variety of tissues and cells. Examples include: body fluid [blood (leukocytes), urine (epithelial cells), saliva, cervical and vaginal secretions . . .] skin, hair roots/follicle, mucus membrane (e.g. buccal or tongue cell scrapings), cervicovaginal cells (from PAP smear, etc.) internal tissue (normal or tumor), chorionic villus tissue, amnionic cells, placental cells, fetal cells, cord blood, sperm or egg.

"Coding sequence" or "DNA coding sequence" refers to those portions of a gene which, taken together, code for a peptide (protein), or for which the nucleic acid itself has function.

A "target polynucleotide" refers to the nucleic acid sequence of interest e.g., the BRCA2 encoding polynucleotide.

"Consensus" means the most commonly occurring in the population.

"Cancer", "tumor" and other similar terms refer to any neoplasm whether benign or malignant, and regardless of whether it has metastisized or the location of the "cancer" or "tumor".

"Substantially complementary to" refers to probe or primer sequences which hybridize to the sequences listed under stringent conditions and/or sequences having sufficient homology with test polynucleotide sequences, such that the allele specific oligonucleotide probe or primers hybridize to the test polynucleotide sequences to which they are complimentary.

"Sequence variation" as used herein refers to any difference in nucleotide sequence between two different oligonucleotide or polynucleotide sequences.

"Polymorphism" as used herein refers to a sequence variation in a gene which is not necessarily associated with pathology.

"Mutation" as used herein refers to an altered genetic sequence which results in the gene coding for a non-functioning protein or a protein with substantially reduced or altered function. Generally, a deleterious mutation is associated with pathology or the potential for pathology.

"Pre-determined sequence variation" as used herein refers to a nucleotide sequence that is designed to be different than the corresponding sequence in a reference nucleotide sequence. A pre-determined sequence variation can be a known mutation in the BRCA2 gene.

"BRCA2 gene" is a group of compounds and refers to the published gene sequences, those appearing in the GENBANK database and the BIC database. Other different sequences include polymorphisms and genetic alterations, especially those which define other haplotypes for the BRCA2 gene. Generally polymorphisms which don't cause an amino acid change or which are naturally occurring (wild types), which are not associated with pathology are also considered the BRCA2 gene. The corresponding nucleotides would then be used even if the nucleotide number differs. While the BRCA2 gene discussed herein is the human BRCA2 gene, the corresponding assays and reagents for the gene in other animals may also be used. The BRCA2 gene includes the coding sequences, non-coding sequences (e.g. introns) and regulatory regions affecting gene expression.

"Allele specific detection assay" as used herein refers to an assay to detect the presence or absence of a predetermined sequence variation in a test polynucleotide or oligonucleotide by annealing the test polynucleotide or oligonucleotide with a polynucleotide or oligonucleotide of predetermined sequence such that differential DNA sequence based techniques or DNA amplification methods discriminate between normal and mutant.

"Sequence variation locating assay" as used herein refers to an assay that detects a sequence variation in a test polynucleotide or oligonucleotide and localizes the position of the sequence variation to a subregion of the test polynucleotide, without necessarily determining the precise base change or position of the sequence variation.

"Region" as used herein generally refers to an area from several nucleotides upstream to several nucleotides downstream from the specific nucleotide mentioned. "Region" also includes the complementary nucleotides on the antisense strand of sample DNA.

"Targeted confirmatory sequencing" as used herein refers to sequencing a polynucleotide in the region wherein a sequence variation has been located by a sequence variation locating assay in order to determine the precise base change and/or position of the sequence variation.

"Probe" includes any oligonucleotide which hybridizes to a BRCA2 or mutant BRCA2 sequence. The probe may be labeled (directly or indirectly) or it may act as a primer such as a PCR primer. The probes of the present invention include the sequences recited and complementary sequences which would aneal to the antisense strand of the sample target DNA. Since both strands of DNA are complementary and mirror images of each other, the complementary version of the mutation is equally unique and indicative of the mutation to be assayed.

Allele Specific Oligonucleotide hybridization is sometimes referred to ASO or the ASO method.

The invention in several of its embodiments includes:

Detection Of Pre-Determined Sequence Variations

Stage I analysis may be used to determine the presence or absence of a pre-determined nucleotide sequence variation; preferably a known mutation or set of known mutations in the test gene. In accordance with the invention, such predetermined sequence variations are detected by allele specific hybridization, a sequence-dependent-based technique which permits discrimination between normal and mutant alleles. An allele specific assay is dependent on the differ-

ential ability of mismatched nucleotide sequences (e.g., normal:mutant) to hybridize with each other, as compared with matching (e.g., normal:normal or mutant:mutant) sequences.

Detection Of Pre-Determined Sequence Variations Using Allele Specific Hydridization

A variety of methods well-known in the art can be used for detection of pre-determined sequence variations by allele specific hybridization. Preferably, the test gene is probed with allele specific oligonucleotides (ASOs); and each ASO contains the sequence of a known mutation. ASO analysis detects specific sequence variations in a target polynucleotide fragment by testing the ability of a specific oligonucleotide probe to hybridize to the target polynucleotide fragment. Preferably, the oligonucleotide contains the mutant sequence (or its complement). The presence of a sequence variation in the target sequence is indicated by hybridization between the oligonucleotide probe and the target fragment under conditions in which an oligonucleotide probe containing a normal sequence does not hybridize to the target fragment. A lack of hybridization between the sequence variant (e.g., mutant) oligonucleotide probe and the target polynucleotide fragment indicates the absence of the specific sequence variation (e.g., mutation) in the target fragment. In a preferred embodiment, the test samples are probed in a standard dot blot format. Each region within the test gene that contains the sequence corresponding to the ASO is individually applied to a solid surface, for example, as an individual dot on a membrane. Each individual region can be produced, for example, as a separate PCR amplification product using methods well-known in the art (see, for example, the experimental embodiment set forth in Mullis, K. B., 1987, U.S. Pat. No. 4,683,202). The use of such a dot blot format is described in detail in the Examples below, detailing the Stage I analysis of the human BRCA2 gene to detect the presence or absence of six different known mutations using six corresponding ASOs.

Membrane-based formats that can be used as alternatives to the dot blot format for performing ASO analysis include, but are not limited to, reverse dot blot, (multiplex amplification assay), and multiplex allele-specific diagnostic assay (MASDA).

In a reverse dot blot format, oligonucleotide or polynucleotide probes having known sequence are immobilized on the solid surface, and are subsequently hybridized with the labeled test polynucleotide sample. In this situation, the primers may be labeled or the NTPs maybe labeled prior to amplification to prepare a labeled test polynucleotide sample. Alternatively, the test polynucleotide sample may be labeled subsequent to isolation and/or synthesis.

In a multiplex format, individual samples contain multiple target sequences within the test gene, instead of just a single target sequence. For example, multiple PCR products each containing at least one of the ASO target sequences are applied within the same sample dot. Multiple PCR products can be produced simultaneously in a single amplification reaction using the methods of Caskey et al., U.S. Pat. No. 5,582,989. The same blot, therefore, can be probed by each ASO whose corresponding sequence is represented in the sample dots.

A MASDA format expands the level of complexity of the multiplex format by using multiple ASOs to probe each blot (containing dots with multiple target sequences). This procedure is described in detail in U.S. Pat. No. 5,589,330 by A. P. Shuber, and in Michalowsky et al., *American Journal of Human Genetics,* 59(4): A272, poster 1573, October 1996, each of which is incorporated herein by reference in its entirety. First, hybridization between the multiple ASO probe and immobilized sample is detected. This method relies on the prediction that the presence of a mutation among the multiple target sequences in a given dot is sufficiently rare that any positive hybridization signal results from a single ASO within the probe mixture hybridizing with the corresponding mutant target. The hybridizing ASO is then identified by isolating it from the site of hybridization and determining its nucleotide sequence.

Suitable materials that can be used in the dot blot, reverse dot blot, multiplex, and MASDA formats are well-known in the art and include, but are not limited to nylon and nitrocellulose membranes.

When the target sequences are produced by PCR amplification, the starting material can be chromosomal DNA in which case the DNA is directly amplified. Alternatively, the starting material can be mRNA, in which case the mRNA is first reversed transcribed into cDNA and then amplified according to the well known technique of RT-PCR (see, for example, U.S. Pat. No. 5,561,058 by Gelfand et al.).

The methods described above are suitable for moderate screening of a limited number of sequence variations. However, with the need in molecular diagnosis for rapid, cost effective large scale screening, technologies have developed that integrate the basic concept of ASO, but far exceed the capacity for mutation detection and sample number. These alternative methods to the ones described above include, but are not limited to, large scale chip array sequence-based techniques. The use of large scale arrays allows for the rapid analysis of many sequence variants. A review of the differences in the application and development of chip arrays is covered by Southern, E. M., *Trends In Genetics,* 12: 110–115 (March 1996) and Cheng et al., *Molecular Diagnosis,* 1:183–200 (September 1996). Several approaches exist involving the manufacture of chip arrays. Differences include, but not restricted to: type of solid support to attach the immobilized oligonucleotides, labeling techniques for identification of variants and changes in the sequence-based techniques of the target polynucleotide to the probe.

A promising methodology for large scale analysis on 'DNA chips' is described in detail in Hacia et al., *Nature Genetics,* 14:441–447, (1996) which is hereby incorporated by reference in its entirety. As described in Hacia et al., high density arrays of over 96,000 oligonucleotides, each 20 nucleotides in length, are immobilized to a single glass or silicon chip using light directed chemical synthesis. Contingent on the number and design of the oligonucleotide probe, potentially every base in a sequence can be interrogated for alterations. Oligonucleotides applied to the chip, therefore, can contain sequence variations that are not yet known to occur in the population, or they can be limited to mutations that are known to occur in the population.

Prior to hybridization with olignucleotide probes on the chip, the test sample is isolated, amplified and labeled (e.g. fluorescent markers) by means well known to those skilled in the art. The test polynucleotide sample is then hybridized to the immobilized oligonucleotides. The intensity of sequence-based techniques of the target polynucleotide to the immobilized probe is quantitated and compared to a reference sequence. The resulting genetic information can be used in molecular diagnosis.

A common, but not limiting, utility of the 'DNA chip' in molecular diagnosis is screening for known mutations. However, this may impose a limitation on the technique by only looking at mutations that have been described in the field. The present invention allows allele specific hybridization analysis be performed with a far greater number of mutations than previously available. Thus, the efficiency and comprehensiveness of large scale ASO analysis will be

broadened, reducing the need for cumbersome end-to-end sequence analysis, not only with known mutations but in a comprehensive manner all mutations which might occur as predicted by the principles accepted, and the cost and time associated with these cumbersome tests will be decreased.

EXAMPLE

Genomic DNA (at least 100 ng) is isolated from white blood cells of a subject with a family history of breast, ovarian or other cancer. Dideoxy sequence analysis is performed following polymerase chain reaction amplification of segments of exon 11.

Exon 11 of the BRCA2 gene is subjected to direct dideoxy sequence analysis by asymmetric amplification using the polymerase chain reaction (PCR) to generate a single stranded product amplified from this DNA sample. Shuldiner, et al., Handbook of Techniques in Endocrine Research, p. 457–486, DePablo, F., Scanes, C., eds., Academic Press, Inc., 1993. Fluorescent dye is attached for automated sequencing using the TAQ DYE TERMINATOR KIT (PERKIN-ELMER cat# 401628). DNA sequencing is performed in both forward and reverse directions on an APPLIED BIOSYSTEMS, INC. (ABI) automated sequencer (Model 373 or 377). The software used for analysis of the resulting data is "SEQUENCE NAVIGATOR" purchased through ABI.

The methods of the invention, which can be used to detect sequence variations in any polynucleotide sample, are demonstrated in the Example set forth in this section, for the purpose of illustration, for one gene in particular, namely, the human BRCA2 gene. The BRCA2 coding sequence is approximately 10,248 base pairs encoding the 3418 amino acid BRCA2 protein.

Designing an Allele Specific Oligonucleotide (ASO) Probe

An allele specific oligonucleotide probe is a short, single stranded polynucleotide that is engineered to hybridize exactly to a target sequence under a given set of conditions. Routinely, ASO probes are designed to contain sequences identical to the normal allele and sequence variation respectively. Hybridization of the probe to the target allows for the discrimination of a variant sample. Under stringent conditions, a probe with a variation as simple as a single-base pair will not hybridize to a normal sequence due to a destabilizing effect of the normal-mutant duplex (Ikuta, S. et al, *Nucleic Acids Research,* 15: 797–811 (1987). For use in this invention, probes were used to discriminate between a wild-type or normal sequence from one that is mutated. Each probe pair contained a polynucleotide sequence that encompassed an area that would identify a selected mutation of the BRCA 2 gene.

The design of an ASO hybridization probe must meet two basic requirements. (*Current Protocols in Human Genetics*, section 9.4, (1995)). First, probes that are used together in the same pool should be around the same length. Although the standard length of a probe is optimally 17 base pairs, the range can be as short as about 14 or as long as about 24. Second, the mismatched region should not be placed at the end of the 17 base pair probe, but approximately in the middle of the sequence, approximately 5–7 bases from the 5' end of the probe. In addition, the placement of a mismatch, in the case of a longer probe, should not be at the end, but at a position that allows strong hybridization and stabilization of the polynucleotide strand. In order to minimize the effects of variations in base composition of the probes, tetramethylammonium chloride is used as in the ASO hybrid's buffer (Shuber, T., U.S. Pat. No. 5,633,134). Conventionally, ASO probes are synthesized on a DNA synthesizer. They can be labeled with isotopic or non-isotopic detection agents using means familiar to those of skill in the art. The process outlined in this application for making and using probes can be applicable for other gene sequences.

Detailed Method For The Detection Of Sequence Variations In Polynucleotides

Isolation of Genomic DNA

White blood cells were collected from the patient and genomic DNA is extracted from the white blood cells according to well-known methods (Sambrook, et al., Molecular Cloning, A Laboratory Manual, 2nd Ed., 1989, Cold Spring Harbor Laboratory Press, at 9.16–9.19).

PCR Amplification for Sequencing

The genomic DNA is used as a template to amplify a separate DNA fragment encompassing the site of the mutation to be tested. The 25 μl PCR reaction contained the following components: 1 μl template (100 ng/μl) DNA, 2.5 μl 10× PCR Buffer (PERKIN-ELMER), 1.5 μl dNTP (2 mM each DATP, dCTP, dGTP, dTTP), 1.5 μl Forward Primer (10 μM), 1.5 μl Reverse Primer (10 μM), 0.5 μl (2.5 U total) AMPLITAQ GOLD™ TAQ DNA POLYMERASE or AMPLITAQ® TAQ DNA POLYMERASE (PERKIN-ELMER), 1.0 to 5.0 μl (25 mM) $MgCl_2$ (depending on the primer) and distilled water (dH_2O) up to 25 μl. All reagents for each exon except the genomic DNA can be combined in a master mix and aliquoted into the reaction tubes as a pooled mixture.

For each exon analyzed, the following control PCRs were set up:

(1) "Negative" DNA control (100 ng placental DNA (SIGMA CHEMICAL CO., St. Louis, Mo.)

(2) Three "no template" controls

PCR for all exons is performed using the following thermocycling conditions:

Temperature	Time	Number of Cycles
95° C.	5 min. (AMPLITAQ) or 10 min. (GOLD)	1
95° C.	30 sec.	} 30 cycles
55° C.	30 sec.	
72° C.	1 min	
72° C.	5 min.	1
4° C.	infinity	1

Quality Control Agarose Gel of PCR Amplification

The quality of the PCR products were examined prior to further analysis by electrophoresing an aliquot of each PCR reaction sample on an agarose gel. 5 μl of each PCR reaction is run on an agarose gel along side a DNA 100BP DNA LADDER (Gibco BRL cat# 15628-019). The electrophoresed PCR products were analyzed according to the following criteria:

Each patient sample must show a single band of the size corresponding the number of base pairs expected from the length of the PCR product from the forward primer to the reverse primer. If a patient sample demonstrates smearing or multiple bands, the PCR reaction must be repeated until a clean, single band is detected. If no PCR product is visible or if only a weak band is visible, but the control reactions with placental DNA template produced a robust band, the patient sample should be re-amplified with 2× as much template DNA.

All three "no template" reactions must show no amplification products. Any PCR product present in these reactions

is the result of contamination. If any one of the "no template" reactions shows contamination, all PCR products should be discarded and the entire PCR set of reactions should be repeated after the appropriate PCR decontamination procedures have been taken.

The optimum amount of PCR product on the gel should be between 50 and 100 ng, which can be determined by comparing the intensity of the patient sample PCR products with that of the DNA ladder. If the patient sample PCR products contain less than 50 to 100 ng, the PCR reaction should be repeated until sufficient quantity is obtained.

DNA Sequencing

For DNA sequencing, double stranded PCR products are labeled with four different fluorescent dyes, one specific for each nucleotide, in a cycle sequencing reaction. With Dye Terminator Chemistry, when one of these nucleotides is incorporated into the elongating sequence it causes a termination at that point. Over the course of the cycle sequencing reaction, the dye-labeled nucleotides are incorporated along the length of the PCR product generating many different length fragments.

The dye-labeled PCR products will separate according to size when electrophoresed through a polyacrylamide gel. At the lower portion of the gel on an ABI automated sequencers, the fragments pass through a region where a laser beam continuously scans across the gel. The laser excites the fluorescent dyes attached to the fragments causing the emission of light at a specific wavelength for each dye. Either a photomultiplier tube (PMT) detects the fluorescent light and converts is into an electrical signal (ABI 373) or the light is collected and separated according to wavelength by a spectrograph onto a cooled, charge coupled device (CCD) camera (ABI 377). In either case the data collection software will collect the signals and store them for subsequent sequence analysis.

PCR products were first purified for sequencing using a QIAQUICK-SPIN PCR PURIFICATION KIT (QIAGEN #28104). The purified PCR products were labeled by adding primers, fluorescently tagged dNTPs and Taq Polymerase FS in an ABI Prism Dye Terminator Cycle Sequencing Kit (PERKIN ELMER/ABI catalog #02154) in a PERKIN ELMER GENEAMP 9600 thermocycler.

The amounts of each component are:

For Samples		For Controls	
Reagent	Volume	Reagent	Volume
Dye mix	8.0 μL	PGEM	2.0 μL
Primer (1.6 mM)	2.0 μL	M13	2.0 μL
PCR product	2.0 μL	Dye mix	8.0 μL
sdH2O	8.0 μL	sdH2O	8.0 μL

The thermocycling conditions were:

Temperature	Time	# of Cycles
96° C.	15 sec.	25
50° C.	5 sec.	
60° C.	4 min.	
4° C.	Infinity	1

The product was then loaded into a gel and placed into an ABI DNA Sequencer (Models 373A & 377) and run. The sequence obtained was analyzed by comparison to the wild type (reference) sequence within the SEQUENCE NAVIGATOR. When a sequence does not align, it indicates a possible mutation. The DNA sequence was determined in both the forward and reverse direction. All results were provided to a second reader for review.

Heterozygous/homozygous point mutations and polymorphisms must be seen in both strands. Frameshift mutations will be seen in both strands and must have clear double peaks in frame shift regions to be so identified.

PCR Amplification for ASO

The genomic DNA is used as a template to amplify a separate DNA fragment encompassing the site of the mutation to be tested. The 50 μl PCR reaction contained the following components: 1 μl template (100 ng/μl) DNA, 5.0 μl 10× PCR Buffer (PERKIN-ELMER), 2.5 μl dNTP (2 mM each DATP, dCTP, dGTP, dTTP), 2.5 μl Forward Primer (10 μM), 2.5 μl Reverse Primer (10 μM), 0.5 μl (2.5 U total) AMPLITAQ® TAQ DNA POLYMERASE or AMPLITAQ GOLD™ DNA POLYMERASE (PERKIN-ELMER), 1.0 to 5.0 μl (25 mM) $MgCl_2$ (depending on the primer) and distilled water (dH_2O) up to 50 μl. All reagents for each exon except the genomic DNA can be combined in a master mix and aliquoted into the reaction tubes as a pooled mixture.

For each exon analyzed, the following control PCRs were set up:

(1) "Negative" DNA control (100 ng placental DNA (SIGMA CHEMICAL CO., St. Louis, Mo.)

(2) Three "no template" controls

PCR for all exons is performed using the following thermocycling conditions:

Temperature	Time	Number of Cycles
95° C.	5 min. (AMPLITAQ) or 10 min. (GOLD)	1
95° C.	30 sec.	30 cycles
55° C.	30 sec.	
72° C.	1 min	
72° C.	5 min.	1
4° C.	infinity	1

The quality control agarose gel of PCR amplification was performed as above.

Binding PCR Products to Nylon Membrane

The PCR products are denatured no more than 30 minutes prior to binding the PCR products to the nylon membrane. To denature the PCR products, the remaining PCR reaction (45 μl) and the appropriate positive control mutant gene amplification product are diluted to 200 μl final volume with PCR Diluent Solution (500 mM NaOH, 2.0 M NaCl, 25 mM EDTA) and mixed thoroughly. The mixture is heated to 95° C. for 5 minutes, and immediately placed on ice and held on ice until loaded onto dot blotter, as described below.

The PCR products are bound to 9 cm by 13 cm nylon ZETA PROBE BLOTTING MEMBRANE (BIO-RAD, Hercules, Calif., catalog number 162-0153) using a BIO-RAD dot blotter apparatus. Forceps and gloves are used at all times throughout the ASO analysis to manipulate the membrane, with care taken never to touch the surface of the membrane with bare hands or latex gloves.

Pieces of 3 MM filter paper [WHATMAN®, Clifton, N.J.] and nylon membrane are pre-wet in 10× SSC prepared fresh from 20× SSC buffer stock. The vacuum apparatus is rinsed thoroughly with dH_2O prior to assembly with the membrane. 100 μl of each denatured PCR product is added to the wells of the blotting apparatus. Each row of the blotting

apparatus contains a set of reactions for a single exon to be tested, including a placental DNA (negative) control, a synthetic oligonucleotide with the desired mutation or a PCR product from a known mutant sample (positive control), and three no template DNA controls.

After applying PCR products, the nylon filter is placed DNA side up on a piece of 3 MM filter paper saturated with denaturing solution (1.5M NaCl, 0.5 M NaOH) for 5 minutes. The membrane is transferred to a piece of 3 MM filter paper saturated with neutralizing solution (1M Tris-HCl, pH 8, 1.5 M NaCl) for 5 minutes. The neutralized membrane is then transferred to a dry 3 MM filter DNA side up, and exposed to ultra-violet light (STRALINKER, STRATAGENE, La Jolla, Calif.) for exactly 45 seconds the fix the DNA to the membrane. This UW crosslinking should be performed within 30 min. of the denaturation/neutralization steps. The nylon membrane is then cut into strips such that each strip contains a single row of blots of one set of reactions for a single exon.

Hybridizing Labeled Oligonucleotides to the Nylon Membrane

Prehybridization

The strip is prehybridized at 52° C. using the HYBAID® (SAVANT INSTRUMENTS, INC., Holbrook, N.Y.) hybridization oven. 2× SSC (15 to 20 ml) is preheated to 52° C. in a water bath. For each nylon strip, a single piece of nylon mesh cut slightly larger than the nylon membrane strip (approximately 1"×5") is pre-wet with 2× SSC. Each single nylon membrane is removed from the prehybridization solution and placed on top of the nylon mesh. The membrane/mesh "sandwich" is then transferred onto a piece of Parafilm. The membrane/mesh sandwich is rolled lengthwise and placed into an appropriate HYBAID® bottle, such that the rotary action of the HYBAID® apparatus caused the membrane to unroll. The bottle is capped and gently rolled to cause the membrane/mesh to unroll and to evenly distribute the 2× SSC, making sure that no air bubbles formed between the membrane and mesh or between the mesh and the side of the bottle. The 2× SSC is discarded and replaced with 5 ml TMAC Hybridization Solution, which contained 3 M TMAC (tetramethyl ammoniumchloride—SIGMA T-3411), 100 mM Na_3PO_4(pH6.8), 1 mM EDTA, 5× Denhardt's (1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA (fraction V)), 0.6% SDS, and 100 μg/ml Herring Sperm DNA. The filter strips were prehybridized at 52° C. with medium rotation (approx. 8.5 setting on the HYBAID® speed control) for at least one hour. Prehybridization can also be performed overnight.

Labeling Oligonucleotides

The DNA sequences of the oligonucleotide probes used to detect the BRCA2 mutations are as follows (for each mutation, a mutant and a normal oligonucleotide must be labeled):

C2192G—normal 5'TGA AGA ACC AAC TTT GT3' SEQ ID NO:3

C2192G—mutant 5'TGA AGA ACG AAC TTT GT3' SEQ ID NO:4

3772delTT—normal 5'GCA AGC AAT TTG AAG GT3' SEQ ID NO:7

3772delTT—mutant 5'GCA AGC AAT GAA GGT AC3' SEQ ID NO:8

C5193G—normal 5'ACT TGT TAC ACA AAT CA3'SEQ ID NO:11

C5193G—mutant 5'ACT TGT TAG ACA AAT CA3' SEQ ID NO:12

5374del4—normal 5'ATT ATT TGT ATG AAA AT3' SEQ ID NO:15

5374del4—mutant 5'ATT ATT TGA AAA TAA TT3' SEQ ID NO:16

6495delGC—normal 5'GAA CTG AGC ATA GTC TT3' SEQ ID NO:19

6495delGC—mutant 5'GAA CTG AAT AGT CTT CA3' SEQ ID NO:20

6909insG—normal 5'CAG AAG CAG TAG AAA TT3' SEQ ID NO:23

6909insG—mutant 5'CAG AAG CAG GTA GAA AT3' SEQ ID NO:24

Each labeling reaction contains 2-μl 5× Kinase buffer (or 1 μl of 10× Kinase buffer), 5 μl gamma-ATP ^{32}P (not more than one week old), 1 μl T4 polynucleotide kinase, 3 μl oligonucleotide (20 μM stock), sterile H_2O to 10 μl final volume if necessary. The reactions are incubated at 37° C. for 30 minutes, then at 65° C. for 10 minutes to heat inactivate the kinase. The kinase reaction is diluted with an equal volume (10 μl) of sterile dH_2O (distilled water).

The oligonucleotides are purified on STE MICRO SELECT-D, G-25 spin columns (catalog no. 5303-356769), according to the manufacturer's instructions. The 20 μl synthetic oligonucleotide eluate is diluted with 80 μl dH_2O (final volume=100 μl). The amount of radioactivity in the oligonucleotide sample is determined by measuring the radioactive counts per minute (cpm). The total radioactivity must be at least 2 million cpm. For any samples containing less than 2 million total, the labeling reaction is repeated.

Hybridization with Mutant Oligonucleotides

Approximately 2–5 million counts of the labeled mutant oligonucleotide probe is diluted into 5 ml of TMAC hybridization solution, containing 40 μl of 20 μM stock of unlabeled normal oligonucleotide. The probe mix is preheated to 52° C. in the hybridization oven. The pre-hybridization solution is removed from each bottle and replaced with the probe mix. The filter is hybridized for 1 hour at 52° C. with moderate agitation. Following hybridization, the probe mix is decanted into a storage tube and stored at −20° C. The filter is rinsed by adding approximately 20 ml of 2× SSC+0.1% SDS at room temperature and rolling the capped bottle gently for approximately 30 seconds and pouring off the rinse. The filter is then washed with 2× SSC+0.1% SDS at room temperature for 20 to 30 minutes, with shaking.

The membrane is removed from the wash and placed on a dry piece of 3 MM WHATMAN filter paper then wrapped in one layer of plastic wrap, placed on the autoradiography film, and exposed for about five hours depending upon a survey meter indicating the level of radioactivity. The film is developed in an automatic film processor.

Control Hybridization with Normal Oligonucleotides

The purpose of this step is to ensure that the PCR products are transferred efficiently to the nylon membrane.

Following hybridization with the mutant oligonucleotide, as described in the Examples above, each nylon membrane is washed in 2× SSC, 0.1% SDS for 20 minutes at 65° C. to melt off the mutant oligonucleotide probes. The nylon strips were then prehybridized together in 40 ml of TMAC hybridization solution for at least 1 hour at 52° C. in a shaking water bath. 2–5 million counts of each of the normal labeled oligonucleotide probes plus 40 μl of 20 μM stock of unlabeled normal oligonucleotide are added directly to the container containing the nylon membranes and the prehybridization solution. The filter and probes are hybridized at 52° C. with shaking for at least 1 hour. Hybridization can be performed overnight, if necessary. The hybridization solution is poured off, and the nylon membrane is rinsed in 2× SSC, 0.1% SDS for 1 minute with gentle swirling by hand.

The rinse is poured off and the membrane is washed in 2× SSC, 0.1% SDS at room temperature for 20 minutes with shaking.

The nylon membrane is removed and placed on a dry piece of 3 MM WHATMAN filter paper. The nylon membrane is then wrapped in one layer of plastic wrap and placed on autoradiography film, and exposure is for at least 1 hour.

For each sample, adequate transfer to the membrane is indicated by a strong autoradiographic hybridization signal. For each sample, an absent or weak signal when hybridized with its normal oligonucleotide, indicates an unsuccessful transfer of PCR product, and it is a false negative. The ASO analysis must be repeated for any sample that did not successfully transfer to the nylon membrane.

Interpreting Results

After hybridizing with mutant oligonucleotides, the results for each exon are interpreted as follows:

TABLE 4A

Result					Interpretation	Action
● (+)	○ (-)	○ NT	○ NT	○ NT	All controls indicate assay is successful	Record results, dark circles are mutation positive, and all others are negative
● (+)	● (-)	○ NT	○ NT	○ NT	Assay not specific, mutant oligonucleotide hybridizing to normal DNA.	Rewash membrane 30 minutes longer at appropriate temp. and re-expose.
○ (+)	○ (-)	○ NT	○ NT	○ NT	Mutant oligonucleotide probe is either washed off or did not label well enough, or PCR product is not transferred to membrane efficiently.	Rehybridize with remaining labeled oligonucleotide. If still no signal, perform normal oligonucleotide hyb. as per the Examples to test transfer of PCR to membrane.
● (+)	○ (-)	▒ NT	▒ NT	▒ NT	Positive and negative controls indicate assay is successful, but PCR is contaminated.	Perform standard clean up procedures for PCR contamination. Repeat assay.

After hybridization with normal oligonucleotides, interpret the results as follows:

TABLE 4B

● (+)	● (-)	○ NT	○ NT	○ NT	Results indicate transfer of PCR products to membrane is successful.	Record results.
● (+)	● (-)	● #1	○ NT	○ NT	○ NT	Results indicate transfer of patient sample #1 is inefficient. May get false negative from this sample.	This sample will have to be transferred to another membrane and the assay repeated.

The sample #1 should be lighter than the controls. Patient samples containing a mutation are generally heterozygous and will hybridize to both the normal and mutant oligonucleotide probes. The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

All references mentioned herein are incorporated by reference.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 24

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGGTACTTTA ATTTTGTCAC TT 22

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGCAGGCATG ACAGAGAA 18

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS

-continued

(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGAAGAACCA ACTTTGT 17

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGAAGAACGA ACTTTGT 17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTCAGATGTT ATTTTCCAAG C 21

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTGTTAAATA ACCAGAAGCA C 21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCAAGCAATT TGAAGGT 17

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCAAGCAATG AAGGTAC 17

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCAAAGACCC TAAAGTACAG 20

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CATCAAATAT TCCTTCTCTA AG 22

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACTTGTTACA CAAATCA 17

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

-continued

ACTTGTTAGA CAAATCA 17

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GAAAATTCAG CCTTAGC 17

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATCAGAATGG TAGGAAT 17

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

-continued

ATTATTTGTA TGAAAAT 17

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ATTATTTGAA AATAATT 17

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TACAGCAAGT GGAAAGC 17

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAGTTTCAGT TTTACCAAT 19

-continued

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GAACTGAGCA TAGTCTT 17

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GAACTGAATA GTCTTCA 17

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ACTTTTTCTG ATGTTCCTGT G 21

-continued

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PRIMER"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TAAAAATAGT GATTGGCAAC A 21

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CAGAAGCAGT AGAAATT 17

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PROBE"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(B) STRAIN: BRCA2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CAGAAGCAGG TAGAAAT 17

We claim:

1. An isolated oligonucleotide wherein the oligonucleotide is capable of detecting a G at nucleotide number 2192 of a BRCA2 gene by specifically hybridizing to the region containing nucleotide number 2192 of the BRCA2 gene.

2. An isolated oligonucleotide having the sequence 5'TGA AGA ACC AAC TTT GT3', SEQ ID NO:3, or the complementary oligonucleotide thereto.

3. An isolated oligonucleotide according to claim 1 having the sequence 5'TGA AGA ACG AAC TTT GT3', SEQ ID NO:4, or the complementary oligonucleotide thereto.

4. The isolated oligonucleotide wherein the oligonucleotide is capable of detecting a deletion of TT at nucleotide number 3772 of a BRCA2 gene by specifically hybridizing to the region containing nucleotide number 3772 of the BRCA2 gene.

5. An isolated oligonucleotide having the sequence 5'GCA AGC AAT TTG AAG GT3', SEQ ID NO:7, or the complementary oligonucleotide thereto.

6. An isolated oligonucleotide according to claim 4 having the sequence 5'GCA AGC AAT GAA GGT AC3', SEQ ID NO:8, or the complementary oligonucleotide thereto.

7. An isolated oligonucleotide wherein the oligonucleotide is capable of detecting a substitution of G for C at nucleotide number 5193 of a BRCA2 gene by specifically hybridizing to the region containing nucleotide number 5193 of the BRCA2 gene.

8. An isolated oligonucleotide having the sequence 5'ACT TGT TAC ACA AAT CA3', SEQ ID NO:11, or the complementary oligonucleotide thereto.

9. An isolated oligonucleotide according to claim 7 having the sequence 5'ACT TGT TAG ACA AAT CA3', SEQ ID NO:12, or the complementary oligonucleotide thereto.

10. An isolated oligonucleotide according to claim 1 wherein the oligonucleotide is capable of detecting a deletion of TATG at nucleotide number 5374 of a BRCA2 gene by specifically hybridizing to the region containing nucleotide number 5374 of the BRCA2 gene.

11. An isolated oligonucleotide having the sequence 5'ATT ATT TGT ATG AAA AT3', SEQ ID NO:15, or the complementary oligonucleotide thereto.

12. An isolated oligonucleotide according to claim 10 having the sequence 5'ATT ATT TGA AAA TAA TT3', SEQ ID NO:16, or the complementary oligonucleotide thereto.

13. An isolated oligonucleotide wherein the oligonucleotide is capable of detecting a deletion of GC at nucleotide number 6495 of a BRCA2 gene by specifically hybridizing to the region containing nucleotide number 6495 of the BRCA2 gene.

14. An isolated oligonucleotide having the sequence 5'GAA CTG AGC ATA GTC TT3', SEQ ID NO:19, or the complementary oligonucleotide thereto.

15. An isolated oligonucleotide according to claim 13 having the sequence 5'GAA CTG AAT AGT CTT CA3', SEQ ID NO:20, or the complementary oligonucleotide thereto.

16. An isolated oligonucleotide wherein the oligonucleotide is capable of detecting an insertion of G at nucleotide number 6909 of a BRCA gene by specifically hybridizing to the region containing nucleotide number 6909 of the BRCA2 gene.

17. An isolated oligonucleotide having the sequence 5'CAG AAG CAG TAG AAA TT3', SEQ ID NO:23, or the complementary oligonucleotide thereto.

18. An isolated oligonucleotide according to claim 16 having the sequence 5'CAG AAG CAG GTA GAA AT3', SEQ ID NO:24, or the complementary oligonucleotide thereto.

19. The isolated oligonucleotide according to any one of claims 1, 4, 7, 10, 13 and 16 further comprising a label bound thereto.

20. The isolated oligonucleotide according to claim 19 wherein the label is selected from the group consisting of a radiolabel, a fluorescent label, a bioluminescent label, a chemiluminescent label, an enzyme label and a ligand label.

21. A pair of isolated oligonucleotide primers which specifically hybridize to the BRCA2 gene, said pair of primers selected from the group consisting of:

BRCA-2-11F: 5'TGG TAC TTT AAT TTT GTC ACT T3' (SEQ ID NO:1), and

BRCA-2-11R: 5'TGC AGG CAT GAC AGA GAA T3' (SEQ ID NO: 2);

BRCA-2-11F: 5'CTC AGA TGT TAT TTT CAA AGC3' (SEQ ID NO: 5); and

BRCA-2-11R: 5'CTG TTA AAT AAC CAG AAG CAC3' (SEQ ID NO: 6);

BRCA-2-11F: 5'GCA AAG ACC CTA AAG TAC AG3' (SEQ ID NO: 9), and

BRCA-2-11R: 5'CAT CAA ATA TTC CTT CTC TAA G3' (SEQ ID NO: 10);

BRCA-2-11F: 5'GAA AAT TCA GCC TTA GC3' (SEQ ID NO: 13), and

BRCA-2-11R: 5'ATC AGA ATG GTA GGA AT3' (SEQ ID NO: 14);

BRCA-2-11F: 5'TAC AGC AAG TGG AAA GC3' (SEQ ID NO: 17), and

BRCA-2-11R: 5'AAG TTT CAG TTT TAC CAA T3' (SEQ ID NO: 18); and

BRCA-2-11F: 5'ACT TTT TCT GAT GTT CCT GTG3' (SEQ ID NO: 21), and

BRCA-2-11R: 5'TAA AAA TAG TGA TTG GCA ACA3' (SEQ ID NO: 22).

22. The pair of isolated oligonucleotide primers according to claim 21, wherein each primer is bound to a label.

23. The pair of primers according to claim 22 wherein each of said label is selected from the group consisting of a radiolabel, a fluorescent label, a bioluminescent label, a chemiluminescent label, an enzyme label and a ligand label.

24. A method for determining the presence or absence of a sequence variation in the BRCA2 gene at nucleotide number 2192, 3772, 5193, 5374, 6495 or 6909 comprising:

(a) performing an allele specific detection assay for the presence or absence of one or more of said sequence variations; and

(b) determining the presence or absence of a sequence variation in the BRCA2 gene in the BRCA2 gene sample at nucleotide number 2192, 3772, 5193, 5374, 6495 or 6909.

25. The method according to claim 24 wherein the said sequence variation is C2192G, 3772delTT, C5193G, 5374del4, 6495delGC or 6909insG.

26. The method of claim 24 wherein the allele specific detection assay is performed as part of a multiplex amplification assay format.

27. The method of claim 24 wherein the allele specific detection assay is performed using a dot blot format, reverse dot blot format, a MASDA format, or a chip array format.

28. The method according to claim 24 further comprising

(a) performing an allele specific detection assay for the presence or absence of one or more reference sequences without said sequence variations.

29. The method according to claim 28 wherein said reference sequence is a BRCA2 coding sequence.

30. The method according to claim 28 wherein said reference sequence is a BRCA2 genomic sequence.

31. The method according to claim 28 wherein said reference sequence is one or more exons of the BRCA2 gene.

32. A method of detecting a predisposition or higher susceptibility to cancer in an individual, comprising:

(a) digesting DNA from an individual to obtain DNA fragments;

(b) separating said DNA fragments;

(c) detecting a DNA fragment containing nucleotide number 2192, 3772, 5193, 5374, 6495 or 6909 of the BRCA2 gene sequence or a sequence variation at nucleotide number 2192, 3772, 5193, 5374, 6495 or 6909 of the BRCA2 gene sequence by sequencing;

(d) comparing the sequence of said fragment with the BRCA2 gene sequence to determine the presence or absence of a sequence variation at nucleotide number 2192, 3772, 5193, 5374, 6495 or 6909, wherein the presence of a sequence variation indicates a predisposition or higher susceptibility to cancer.

33. A method according to claim 32 further comprising amplifying said DNA fragments prior to the detecting step (c).

34. A method according to claim 32 wherein the DNA fragment containing the sequence variation is amplified with an oligonucleotide primer having a sequence of:
5'TGG TAC TTT AAT TTT GTC ACT T3' SEQ ID NO:1,
5'TGC AGG CAT GAC AGA GAA T3' SEQ ID NO:2,
5'CTC AGA TGT TAT TTT CCA AGC3' SEQ ID NO:5,
5'CTG TTA AAT AAC CAG AAG CAC3' SEQ ID NO:6,
5'GCA AAG ACC CTA AAG TAC AG3' SEQ ID NO:9,
5'CAT CAA ATA TTC CTT CTC TAA G3' SEQ ID NO:10,
5'GAA AAT TCA GCC TTA GC3' SEQ ID NO:13,
5'ATC AGA ATG GTA GGA AT3' SEQ ID NO:14,
5'TAC AGC AAG TGG AAA GC3' SEQ ID NO:17,
5'AAG TTT CAG TTT TAC CAA T3' SEQ ID NO:18,
5'ACT TTT TCT GAT GTT CCT GTG3' SEQ ID NO:21,
5'TAA AAA TAG TGA TTG GCA ACA3' SEQ ID NO:22 or a sequence capable of specific hybridization to and initiation of DNA synthesis on a complementary oligonucleotide or polynucleotide.

35. A method according to claim 34 wherein said oligonucleotide primer is labeled with a radiolabel, a fluorescent label a bioluminescent label, a chemiluminescent label, an enzyme label, or a ligand label.

36. A method of detecting a predisposition or higher susceptibility to cancer in an individual, comprising:

(a) digesting DNA from said individual to obtain DNA fragments,

(b) separating said DNA fragments obtained from said digestion,

(c) subjecting said DNA fragments to hybridization with an allele specific oligonucleotide having a nucleotide sequence capable of specifically hybridizing to a polynucleotide having a sequence variation at nucleotide number 2192, 3772, 5193, 5374, 6495 or 6909 of the BRCA2 gene sequence, thereby determining the absence or presence of said sequence variation in the BRCA2 gene of said individual, and

(d) correlating the presence of said sequence variation with a predisposition or higher susceptibility to cancer.

37. A method according to claim 36 herein said allele specific oligonucleotide is:
5'TGA AGA ACC AAC TTT GT3' SEQ ID NO:3,
5'TGA AGA ACG AAC TTT GT3' SEQ ID NO:4,
5'GCA AGC AAT TTG AAG GT3' SEQ ID NO:7,
5'GCA AGC AAT GAA GGT AC3' SEQ ID NO:8,
5'ACT TGT TAC ACA AAT CA3' SEQ ID NO:11,
5'ACT TGT TAG ACA AAT CA3' SEQ ID NO:12,
5'ATT ATT TGT ATG AAA AT3' SEQ ID NO:15,
5'ATT ATT TGA AAA TAA TT3' SEQ ID NO:16,
5'GAA CTG AGC ATA GTC TT3' SEQ ID NO:19,
5'GAA CTG AAT AGT CTT CA3' SEQ ID NO:20,
5'CAG AAG CAG TAG AAA TT3' SEQ ID NO:23, or
5'CAG AAG CAG GTA GAA AT3' SEQ ID NO:24.

38. A method according to claim 36 further comprising amplifying said DNA fragment prior to sequencing.

39. A method according to claim 36 wherein said oligonucleotide is labeled with a radiolabel, a fluorescent label, a bioluminescent label, a chemiluminescent label, an enzyme label, or a ligand label.

40. A kit comprising a carrier means being compartmentalized to receive in close confinement one or more container means, and at least one container means,

wherein said at least one container means contains the oligonucleotide of any one of claims 1, 4, 7, 10, 13, 16.

41. The kit according to claim 40 further comprising at least one container means containing:
BRCA-2-11F: 5'TGG TAC TTT AAT TTT GTC ACT T3' (SEQ ID NO:1),
BRCA-2 11R: 5'TGC AGG CAT GAC AGA GAA T3' (SEQ ID NO: 2),
BRCA-2-11F: 5'CTC AGA TGT TAT TTT CAA AGC3' (SEQ ID NO:5),
BRCA-2-11R: 5'CTG TTA AAT AAC CAG AAG CAC3' (SEQ ID NO: 6),
BRCA-2-11F: 5'GCA AAG ACC CTA AAG TAC AG3' (SEQ ID NO:9),
BRCA-2-11R: 5'CAT CAA ATA TTC CTT CTC TAA G3' (SEQ ID NO: 10),
BRCA-2-11F: 5'GAA AAT TCA GCC TTA GC3' (SEQ ID NO: 13),
BRCA-2-11R: 5'ATC AGA ATG GTA GGA AT3' (SEQ ID NO:14),
BRCA-2-11F: 5'TAC AGC AAG TGG AAA GC3' (SEQ ID NO: 17),
BRCA-2-11R: 5'AAG TTT CAG TTT TAC CAA T3' (SEQ ID NO:18),
BRCA-2-11F: 5'ACT TTT TCT GAT GTT CCT GTG3' (SEQ ID NO: 21), or
BRCA-2-11R: 5'TAA AAA TAG TGA TTG GCA ACA3' (SEQ ID NO: 22).

42. The kit according to claim 40 further comprising at least one container means containing a pair of isolated oligonucleotide primers which specifically hybridize to the BRCA2 gene, one of which can effectively hybridize to exon 11 of the BRCA2 gene, and the other can effectively hybridize to either exon 11 or one of the two intron regions flanking exon 11.

43. A kit comprising a carrier means being compartmentalized to receive in close confinement one or more container means, and at least one container means,

wherein at least one container means contains the pair of oligonucleotide primers of claim 21.

44. A method of determining whether a C2192G, 3772delTT, C5193G, 5374del4, 6495delGC, or 6909insG mutation is present in a BRCA2 gene comprising sequencing at least a portion of the BRCA2 gene containing either:

a sequence complementary to SEQ ID NO:4, SEQ ID NO:8, SEQ ID NO:12, SEQ ID NO:16:, SEQ ID NO:20 or SEQ ID NO:24, or an isolated DNA sequence while in the complement thereof, or

at least one mutation from the list: C2192G, 3772delTT, C5193G, 5374del4, 6495delGC or 6909insG.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,051,379

DATED : April 18, 2000

INVENTOR(S) : Lescallett et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims:

Column 39, line 37: "according to claim 1" should be deleted;

Column 40, line 42: "6909" should read -- 6909, --;

Column 40, line 45: "variations; and" should read -- variations in the BRCA2 gene; and --;

Column 40, line 47: "in the BRCA2 gene" (second occurrence) should be deleted;

Column 40, line 50: "the" should be deleted;

Column 41, line 54: "sequence variation" should read --sequence contained within the BRCA2 gene sequence or a polynucleotide having a sequence variation--;

Column 41, line 61: "herein" should read -- wherein --;

Column 42, line 19: "containing:" should read -- containing an isolated nucleotide primer comprising the sequence: --;

Column 42, lines 56–57: "C2192G, 3772delTT, C5193G, 5374del4, 6495delGC, or 6909insG" should be deleted;

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,051,379

DATED : April 18, 2000

INVENTOR(S) : Lescallett et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 42, line 63: "while in" should read -- which is --;

Signed and Sealed this

Eighth Day of May, 2001

Attest:

NICHOLAS P. GODICI

Attesting Officer

Acting Director of the United States Patent and Trademark Office

United States Patent [19]

Mullis et al.

[11] **Patent Number: 4,683,195**

[45] **Date of Patent: * Jul. 28, 1987**

[54] **PROCESS FOR AMPLIFYING, DETECTING, AND/OR-CLONING NUCLEIC ACID SEQUENCES**

[75] Inventors: **Kary B. Mullis,** Kensington; **Henry A. Erlich,** Oakland; **Norman Arnheim,** Woodland Hills; **Glenn T. Horn,** Emeryville; **Randall K. Saiki,** Richmond; **Stephen J. Scharf,** Berkeley, all of Calif.

[73] Assignee: **Cetus Corporation,** Emeryville, Calif.

[*] Notice: The portion of the term of this patent subsequent to Jul. 28, 2004 has been disclaimed.

[21] Appl. No.: **828,144**

[22] Filed: **Feb. 7, 1986**

Related U.S. Application Data

[60] Continuation-in-part of Ser. No. 824,044, Jan. 30, 1986, abandoned, which is a division of Ser. No. 791,308, Oct. 25, 1985, which is a continuation-in-part of Ser. No. 716,975, Mar. 28, 1985, abandoned.

[51] **Int. Cl.**[4] **C12Q 1/68;** C12P 19/34; C12N 1/00; C12N 15/00; G01N 33/48; G01N 33/00; G01N 33/566; G01N 33/564; C07H 21/02; C07H 21/04

[52] **U.S. Cl.** **435/6;** 435/91; 435/172.3; 435/317; 436/63; 436/94; 436/501; 436/508; 536/27; 536/28; 536/29; 935/17; 935/18; 935/76; 935/77; 935/78

[58] **Field of Search** 435/91, 172.3, 317, 435/6; 536/27, 28, 29; 935/17, 18, 78, 77, 76; 436/63, 94, 501, 508

[56] **References Cited**

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Salser, "Cloning cDNA sequences: A general Technique for Propagating Eukaryotic Gene Sequences in Bacterial Cells", in Genetic Engineering, 1978, Charrabarty (ed.), CRC Press, Inc. Boca Raton, Fla., pp. 53-81.

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Primary Examiner—James Martinell
Attorney, Agent, or Firm—Janet E. Hasak; Albert P. Halluin

[57] **ABSTRACT**

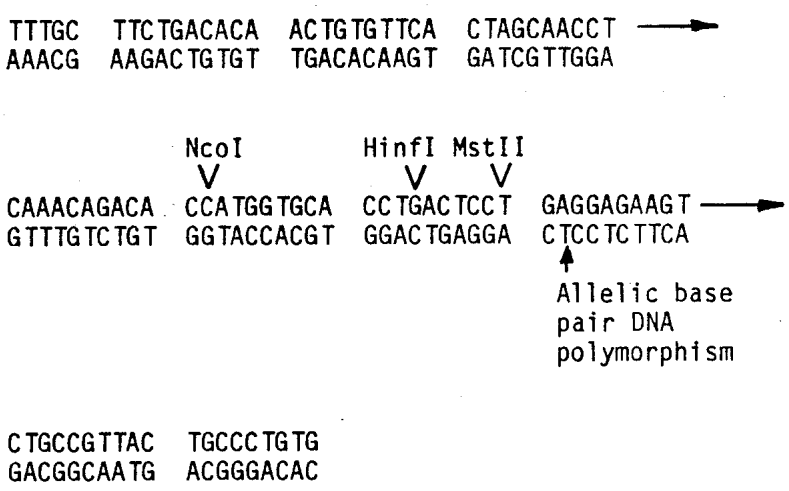
The present invention is directed to a process for amplifying and detecting any target nucleic acid sequence contained in a nucleic acid or mixture thereof. The process comprises treating separate complementary strands of the nucleic acid with a molar excess of two oligonucleotide primers, extending the primers to form complementary primer extension products which act as templates for synthesizing the desired nucleic acid sequence, and detecting the sequence so amplified. The steps of the reaction may be carried out stepwise or simultaneously and can be repeated as often as desired.

In addition, a specific nucleic acid sequence may be cloned into a vector by using primers to amplify the sequence, which contain restriction sites on their non-complementary ends, and a nucleic acid fragment may be prepared from an existing shorter fragment using the amplification process.

26 Claims, 12 Drawing Figures

FIG.1

Double-Stranded 94-bp Sequence



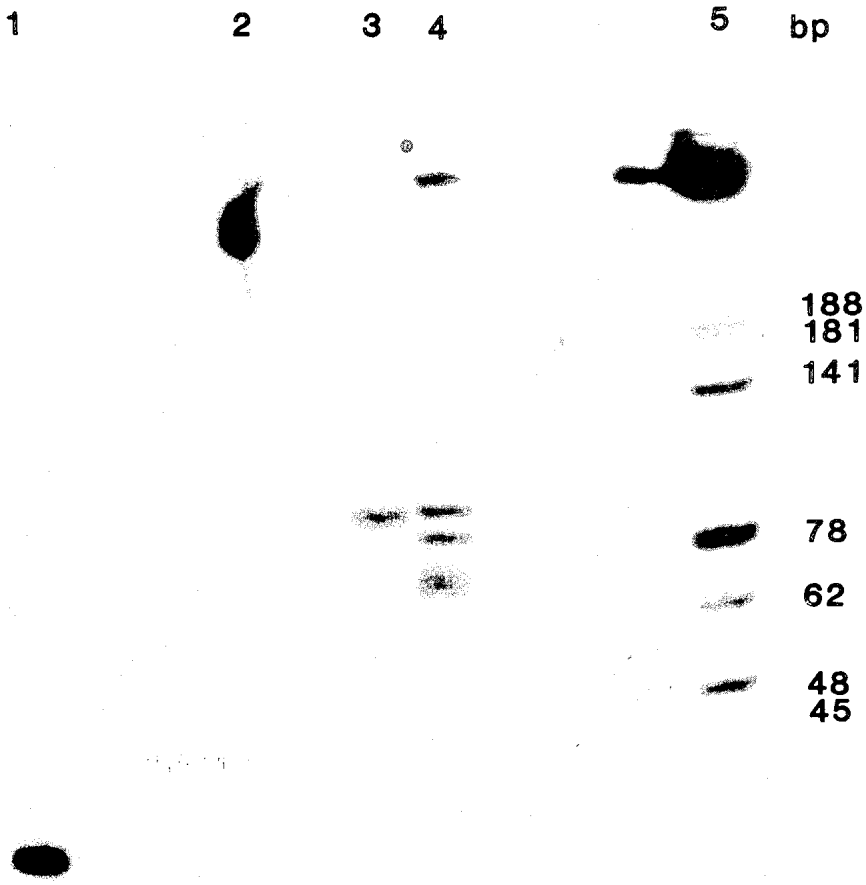


FIG.2

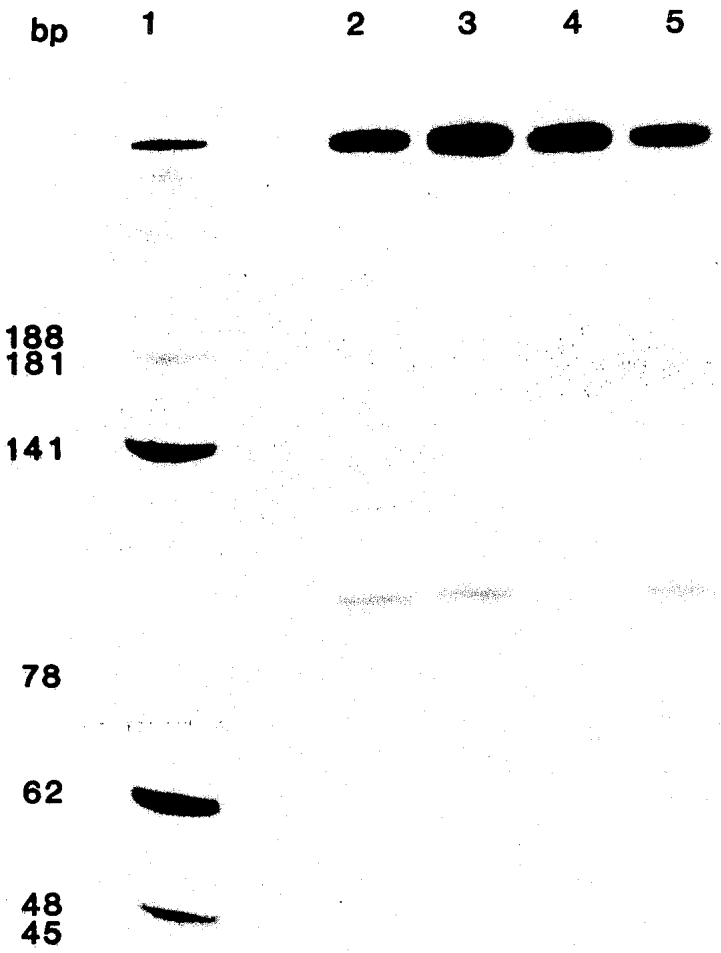


FIG.3

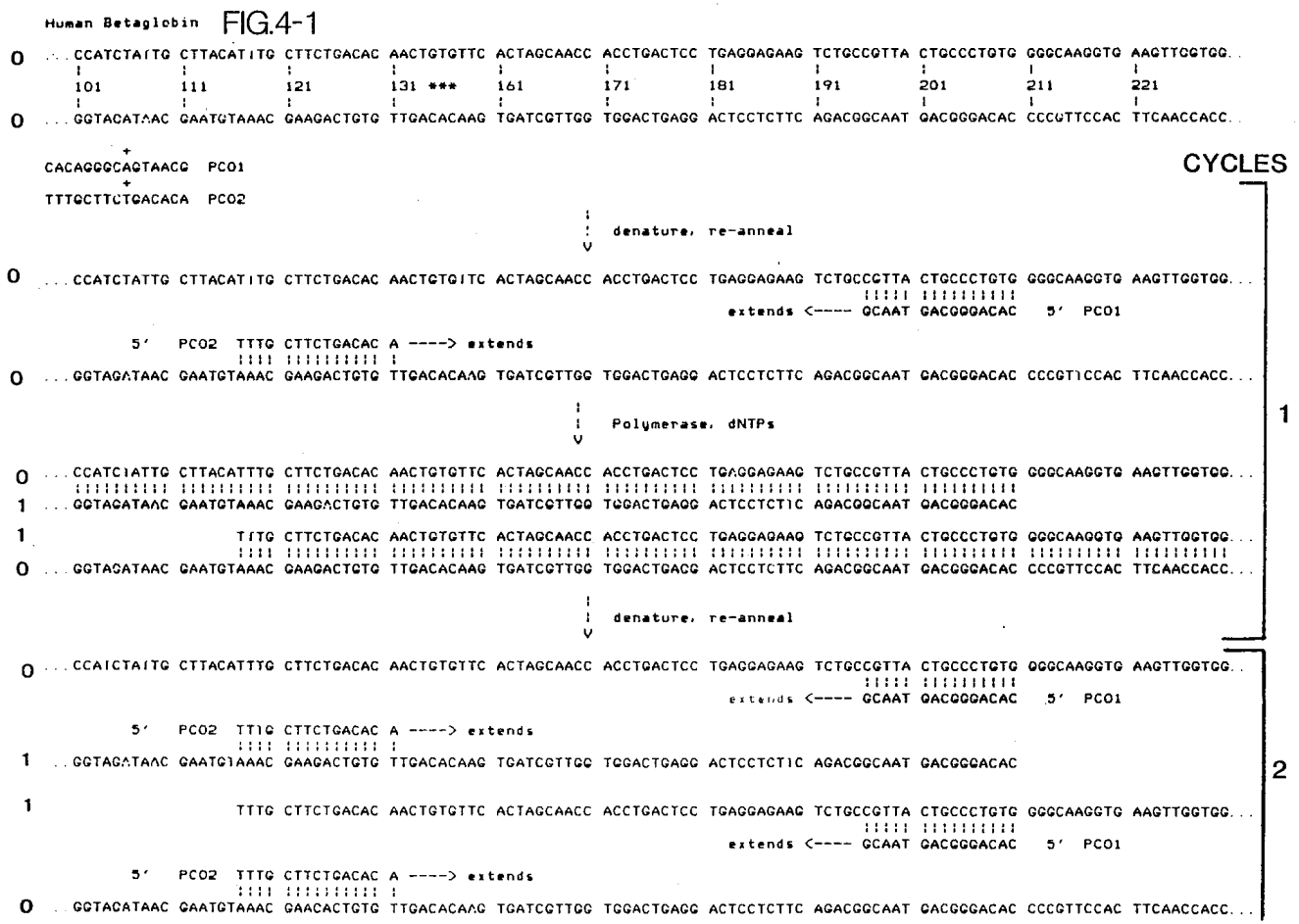

Human Betaglobin
FIG.4-1
CACAGGGCAGTAACG PCO1
TTTGCTTCTGACACA PCO2
denature, re-anneal
Polymerase, dNTPs
denature, re-anneal
CYCLES
1
2
5' PCO2 TTTG CTTCTGACAC A ----> extends
extends <---- GCAAT GACGGGACAC 5' PCO1

FIG.4-2

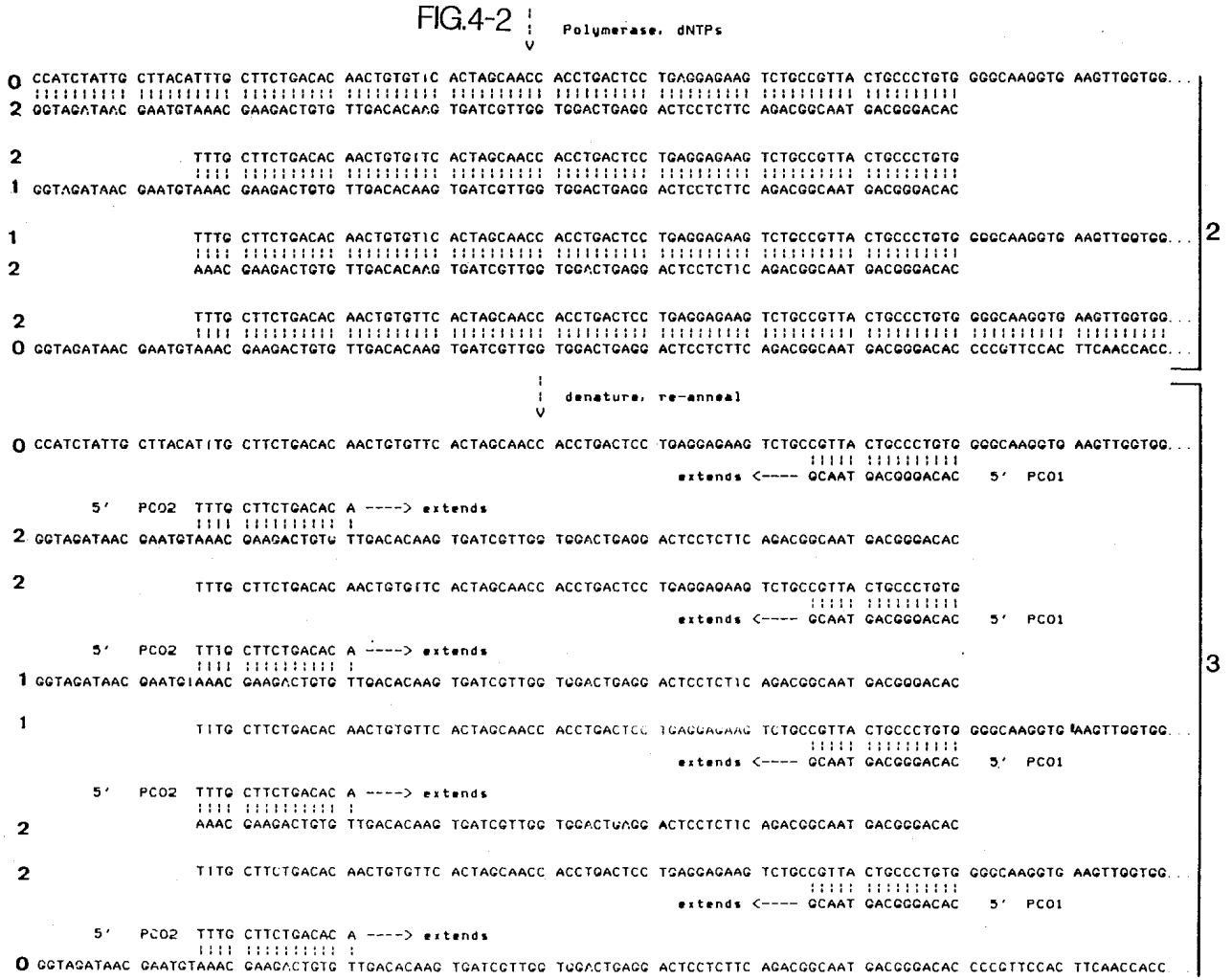
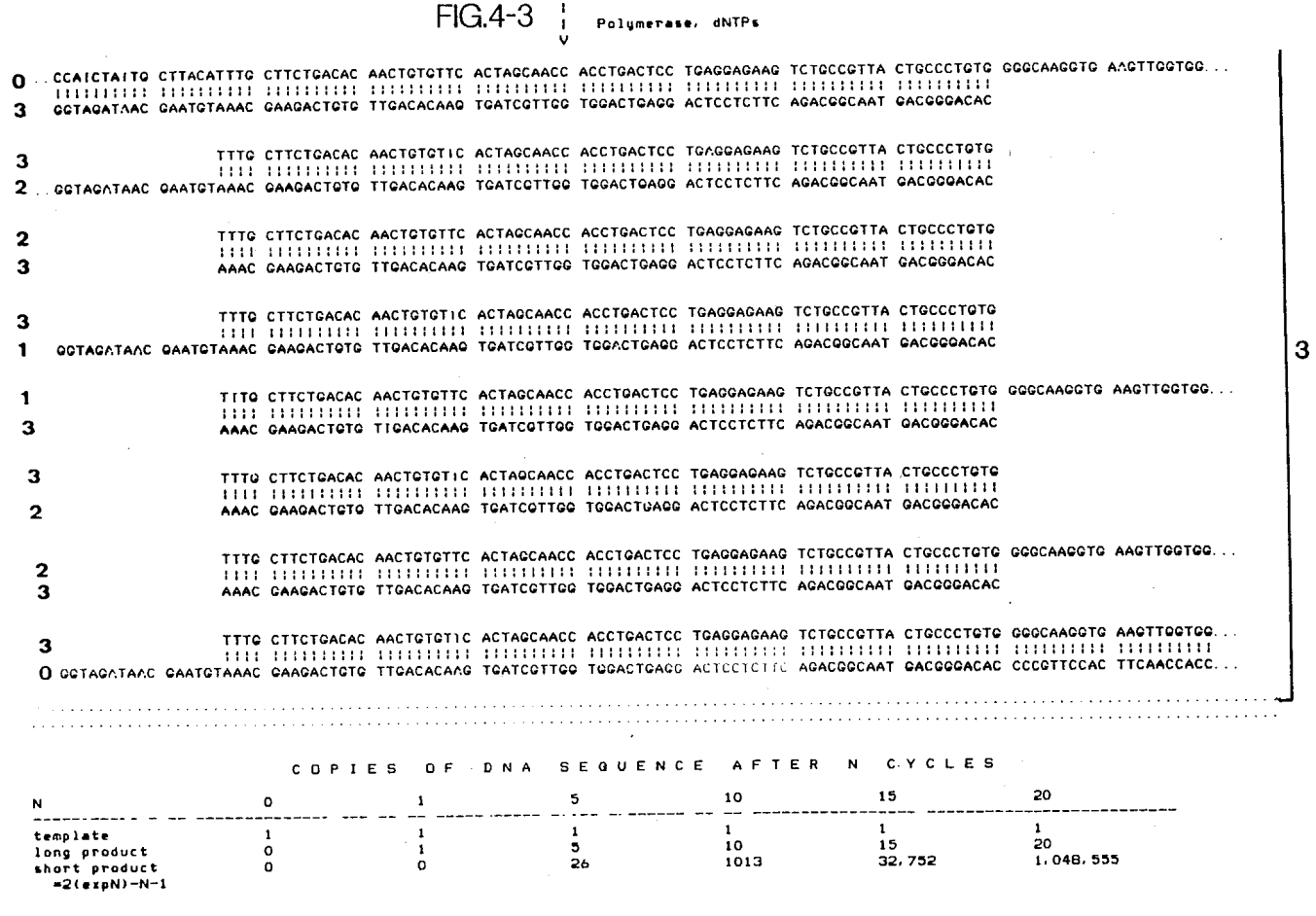

Polymerase, dNTPs
denature, re-anneal
extends
5' PCO2
5' PCO1
2
3

FIG.4-3



COPIES OF DNA SEQUENCE AFTER N CYCLES

N	0	1	5	10	15	20
template	1	1	1	1	1	1
long product	0	1	5	10	15	20
short product =2(expN)-N-1	0	0	26	1013	32,752	1,048,555

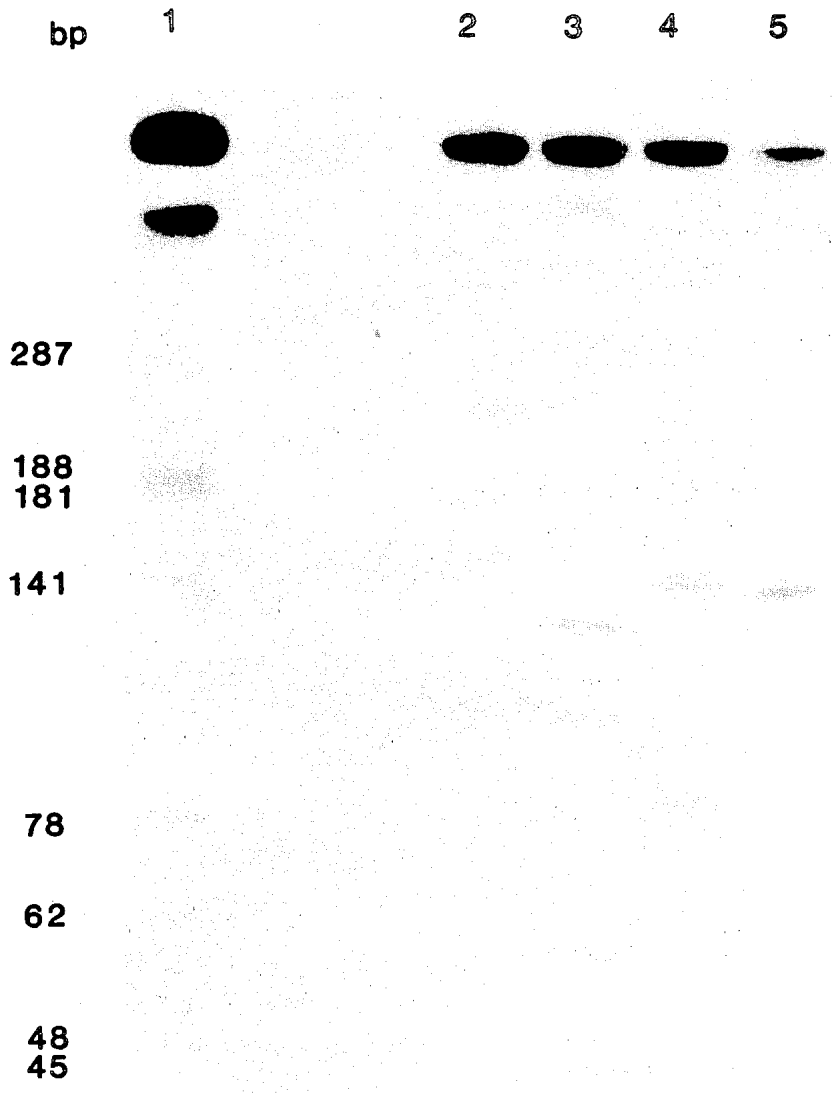
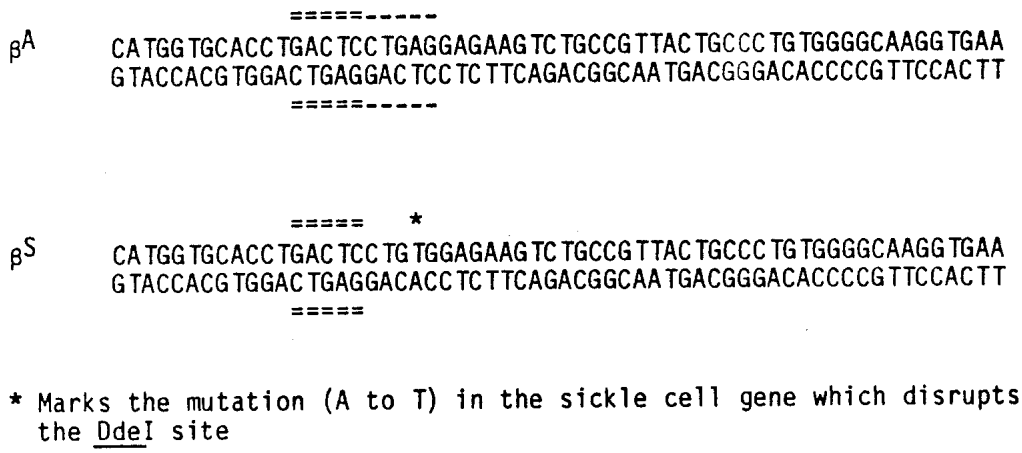
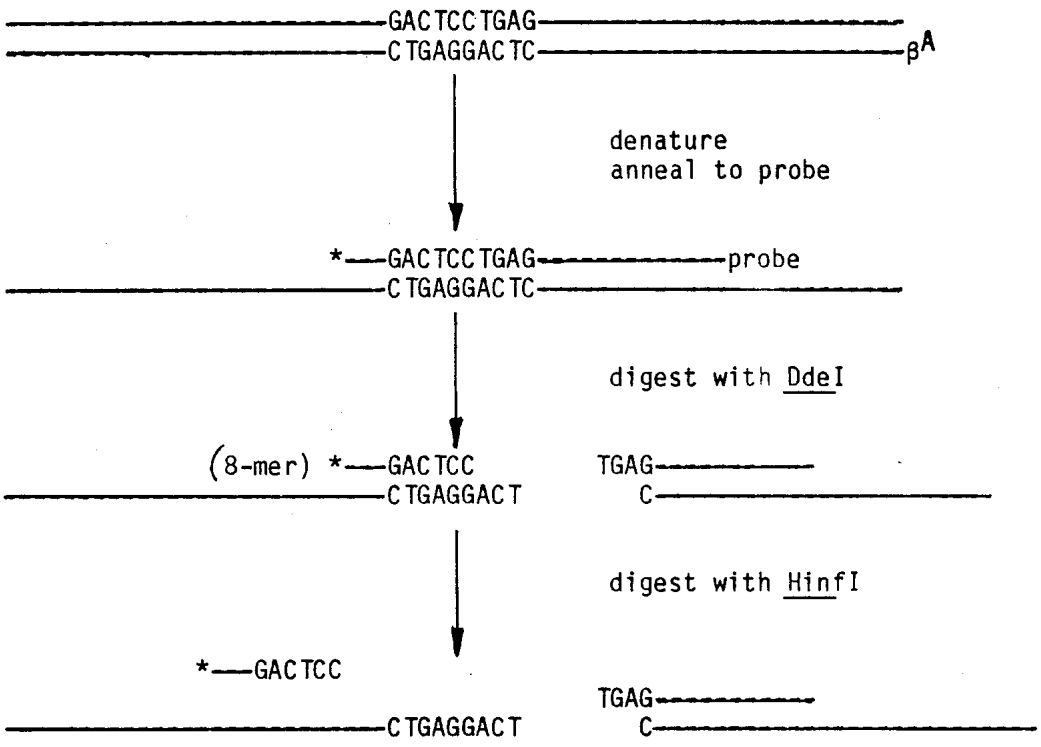


FIG.5

FIG.6



* Marks the mutation (A to T) in the sickle cell gene which disrupts the DdeI site



* is label

FIG.7

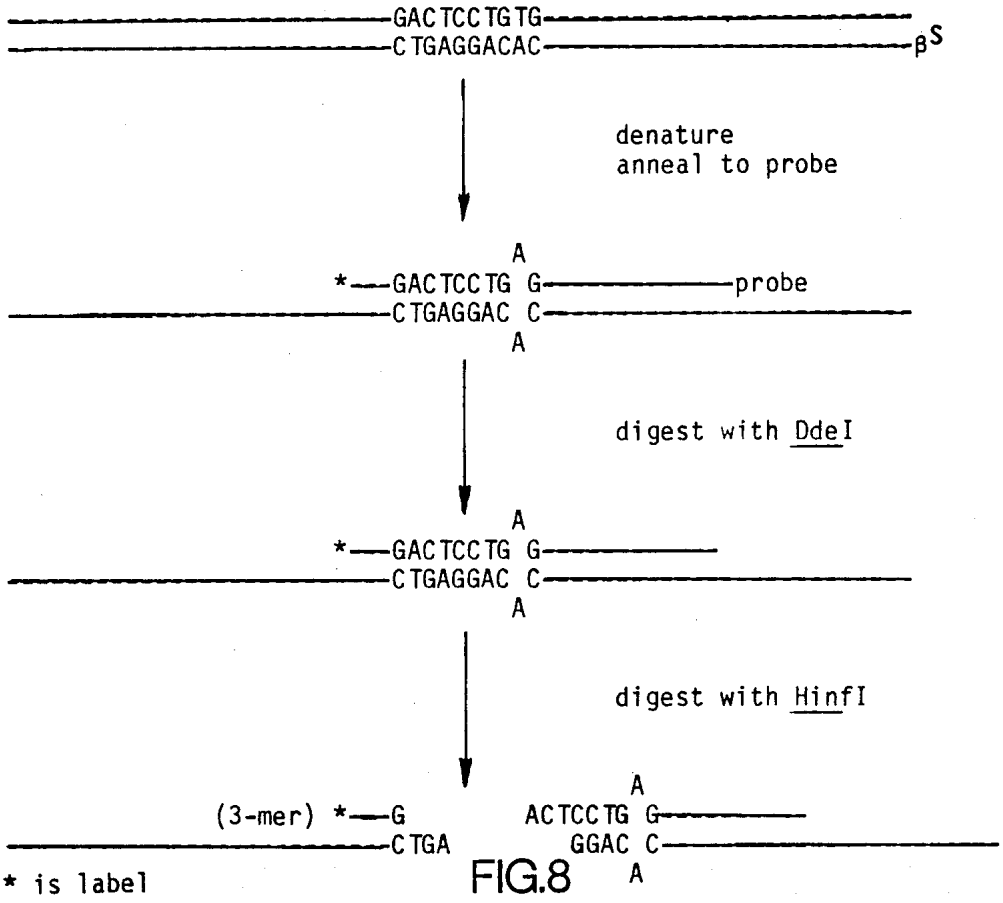

GACTCCTGTG
CTGAGGACAC
βS
denature
anneal to probe
A
*—GACTCCTG G
probe
CTGAGGAC C
A
digest with DdeI
A
*—GACTCCTG G
CTGAGGAC C
A
digest with HinfI
A
(3-mer) *—G
ACTCCTG G
CTGA
GGAC C
A
* is label

FIG.8

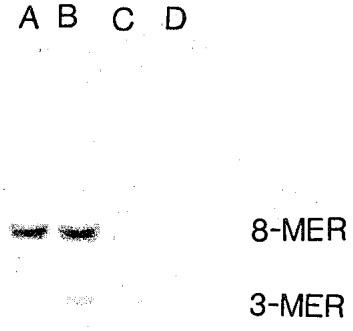
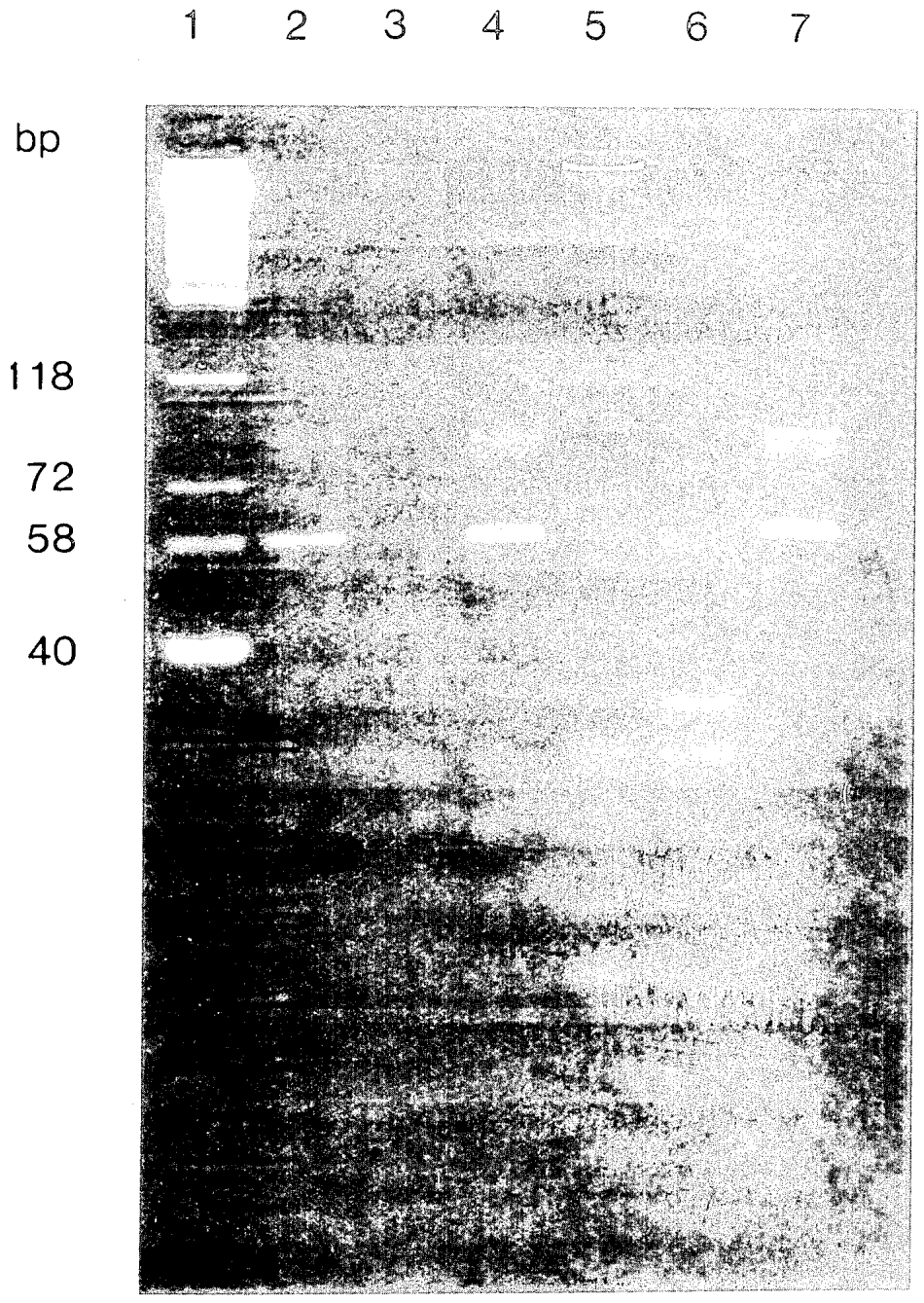


FIG.9

FIG. 10



PROCESS FOR AMPLIFYING, DETECTING, AND/OR-CLONING NUCLEIC ACID SEQUENCES

BACKGROUND OF THE INVENTION

This application is a continuation-in-part application of copending U.S. Ser. No. 824,044 filed Jan. 30, 1986, now abandoned, which is a divisional application of copending U.S. Ser. No. 791,308 filed Oct. 25, 1985, which is a continuation-in-part application of copending U.S. application Ser. No. 716,975 filed Mar. 28, 1985, now abandoned.

FIELD OF THE INVENTION

The present invention relates to a process for amplifying existing nucleic acid sequences if they are present in a test sample and detecting them if present by using a probe. More specifically, it relates to a process for producing any particular nucleic acid sequence from a given sequence of DNA or RNA in amounts which are large compared to the amount initially present so as to facilitate detection of the sequences. The DNA or RNA may be single- or double-stranded, and may be a relatively pure species or a component of a mixture of nucleic acids. The process of the invention utilizes a repetitive reaction to accomplish the amplification of the desired nucleic acid sequence.

DESCRIPTION OF RELATED DISCLOSURES

For diagnostic applications in particular, the target nucleic acid sequence may be only a small portion of the DNA or RNA in question, so that it may be difficult to detect its presence using nonisotopically labeled or end-labeled oligonucleotide probes. Much effort is being expended in increasing the sensitivity of the probe detection systems, but little research has been conducted on amplifying the target sequence so that it is present in quantities sufficient to be readily detectable using currently available methods.

Several methods have been described in the literature for the synthesis of nucleic acids de novo or from an existing sequence. These methods are capable of producing large amounts of a given nucleic acid of completely specified sequence.

One known method for synthesizing nucleic acids de novo involves the organic synthesis of a nucleic acid from nucleoside derivatives. This synthesis may be performed in solution or on a solid support. One type of organic synthesis is the phosphotriester method, which has been utilized to prepare gene fragments or short genes. In the phosphotriester method, oligonucleotides are prepared which can then be joined together to form longer nucleic acids. For a description of this method, see Narang, S. A., et al., *Meth. Enzymol.*, 68, 90 (1979) and U.S. Pat. No. 4,356,270. The patent describes the synthesis and cloning of the somatostatin gene.

A second type of organic synthesis is the phosphodiester method, which has been utilized to prepare a tRNA gene. See Brown, E. L., et al., *Meth. Enzymol.*, 68, 109 (1979) for a description of this method. As in the phosphotriester method, the phosphodiester method involves synthesis of oligonucleotides which are subsequently joined together to form the desired nucleic acid.

Although the above processes for de novo synthesis may be utilized to synthesize long strands of nucleic acid, they are not very practical to use for the synthesis of large amounts of a nucleic acid. Both processes are laborious and time-consuming, require expensive equipment and reagents, and have a low overall efficiency. The low overall efficiency may be caused by the inefficiencies of the synthesis of the oligonucleotides and of the joining reactions. In the synthesis of a long nucleic acid, or even in the synthesis of a large amount of a shorter nucleic acid, many oligonucleotides would need to be synthesized and many joining reactions would be required. Consequently, these methods would not be practical for synthesizing large amounts of any desired nucleic acid.

Methods also exist for producing nucleic acids in large amounts from small amounts of the initial existing nucleic acid. These methods involve the cloning of a nucleic acid in the appropriate host system, where the desired nucleic acid is inserted into an appropriate vector which is used to transform the host. When the host is cultured the vector is replicated, and hence more copies of the desired nucleic acid are produced. For a brief description of subcloning nucleic acid fragments, see Maniatis, T., et al., *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory, pp. 390–401 (1982). See also the techniques described in U.S. Pat. Nos. 4,416,988 and 4,403,036.

A third method for synthesizing nucleic acids, described in U.S. Pat. No. 4,293,652, is a hybrid of the above-described organic synthesis and molecular cloning methods. In this process, the appropriate number of oligonucleotides to make up the desired nucleic acid sequence is organically synthesized and inserted sequentially into a vector which is amplified by growth prior to each succeeding insertion.

The present invention bears some similarity to the molecular cloning method; however, it does not involve the propagation of any organism and thereby avoids the possible hazards or inconvenience which this entails. The present invention also does not require synthesis of nucleic acid sequences unrelated to the desired sequence, and thereby the present invention obviates the need for extensive purification of the product from a complicated biological mixture.

SUMMARY OF THE INVENTION

The present invention resides in a process for amplifying one or more specific nucleic acid sequences present in a nucleic acid or mixture thereof using primers and agents for polymerization and then detecting the amplified sequence. The extension product of one primer when hybridized to the other becomes a template for the production of the desired specific nucleic acid sequence, and vice versa, and the process is repeated as often as is necessary to produce the desired amount of the sequence. This method is expected to be more efficient than the methods described above for producing large amounts of nucleic acid from a target sequence and to produce such nucleic acid in a comparatively short period of time. The present method is especially useful for amplifying rare species of nucleic acid present in a mixture of nucleic acids for effective detection of such species.

More specifically, the present invention provides a process for detecting the presence or absence of at least one specific nucleic acid sequence in a sample containing a nucleic acid or mixture of nucleic acids, or distinguishing between two different forms of sequences in said sample, wherein the sample is suspected of contain-

ing said sequence or sequences, which process comprises:

(a) treating the sample with one oligonucleotide primer for each strand of each different specific sequence suspected of being present in the sample, under hybridizing conditions such that for each strand of each different sequence to be detected an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primer or primers are selected so as to be substantially complementary to each strand of each specific sequence such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer;

(b) treating the sample under denaturing conditions to separate the primer extension products from their templates if the sequence or sequences to be detected are present;

(c) treating the sample with oligonucleotides primers such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, resulting in amplification of the specific nucleic acid sequence or sequences if present;

(d) adding to the product of step (c) a labeled probe capable of hybridizing to said sequence being detected or a mutation thereof; and

(e) determining whether said hybridization has occurred.

The steps (a)–(c) may be conducted sequentially or simultaneously. In addition, steps (b) and (c) may be repeated until the desired level of sequence amplification is obtained.

In other embodiments the invention relates to diagnostic kits for the detection of at least one specific nucleic acid sequence in a sample containing one or more nucleic acids at least one of which nucleic acid is suspected of containing said sequence, which kit comprises, in packaged form, a multicontainer unit having

(a) one container for each oligonucleotide primer for each strand of each different sequence to be detected, which primer or primers are substantially complementary to each strand of each specific nucleic acid sequence such that an extension product synthesized from one primer, when it is separated from its complement, can serve as a template for the synthesis of the extension product of the other primer;

(b) a container containing an agent for polymerization;

(c) a container for each of four different nucleoside triphosphates;

(d) a container containing a probe capable of detecting the presence of said sequence in said sample; and

(e) a container containing means for detecting hybrids of said probe and said sequence.

In yet another embodiment, the invention relates to a process for cloning into a vector a specific nucleic acid sequence contained in a nucleic acid or a mixture of nucleic acids, which process comprises:

(a) treating the nucleic acid(s) with one oligonucleotide primer for each strand of each different specific sequence being amplified, under conditions such that for each strand of each different sequence being amplified an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primer or primers are selected so as to be substantially complementary to each strand of each specific sequence such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein said primer or primers each contain a restriction site on its 5′ end which is the same as or different from the restriction site(s) on the other primer(s);

(b) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

(c) treating the single-stranded molecules generated from step (b) with oligonucleotide primers such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, wherein depending on the particular sequence being amplified, steps (a) and (c) are carried out in the presence of from 0 up to an effective amount of dimethylsulfoxide or at a temperature of up to about 45° C.;

(d) adding to the product of step (c) a restriction enzyme for each of said restriction sites to obtain cleaved products in a restriction digest; and

(e) ligating the cleaved product(s) into one or more cloning vectors.

In yet another embodiment, the invention herein relates to a process for synthesizing a nucleic acid fragment from an existing nucleic acid fragment having fewer nucleotides than the fragment being synthesized and two oligonucleotide primers, wherein the nucleic acid being synthesized is comprised of a left segment, a core segment and a right segment, and wherein the core segment represents at least substantially the nucleotide sequence of said existing nucleic acid fragment, and the right and left segments represent the sequence nucleotide present in the 5′ ends of the two primers, the 3′ ends of which are complementary or substantially complementary to the 3′ ends of the single strands produced by separating the strands of said existing nucleic acid fragment, which process comprises:

(a) treating the strands of said existing fragment with two oligonucleotide primers under condition such that an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primers are selected so as to be substantially complementary to the 3′ end of each strand of said existing fragment such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein each primer contains, at its 5′ end, a sequence of nucleotides which are not complementary to said existing fragment and which correspond to the two ends of the nucleic acid fragment being synthesized;

(b) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

(c) treating the single-stranded molecules generated from step (b) with the primers of step (a) under conditions such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template so as to produce two intermediate double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ end of one of the oligonucleotide primers, and two full-length double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ ends of both of the oligonucleotide primers;

(d) repeating steps (b) and (c) for a sufficient number of times to produce the full-length double-stranded molecule in an effective amount;

(e) treating the strands of the product of step (d) with two primers so as to lengthen the product of step (d) on both ends; and

(f) repeating steps (a)–(d) using the product of step (d) as the core fragment and two oligonucleotide primers which are complementary or substantially complementary to the 3′ ends of the single strands produced by separating the strands of the product of step (d).

The core fragment may be obtained by the steps comprising:

(a) reacting two oligonucleotides, each of which contain at their 3′ ends a nucleotide sequence which is complementary to the other oligonucleotide at its 3′ end, and which are non-complementary to each other at their 5′ ends, with an agent for polymerization and four nucleoside triphosphates under conditions such that an extension product of each oligonucleotide is synthesized which is complementary to each nucleic acid strand;

(b) separating the extension products from the templates on which they were synthesized to produce single-stranded molecules; and

(c) treating the single-stranded molecules generated from step (b) with the oligonucleotides of step (a) under conditions such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, resulting in amplification of the core fragment.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** illustrates a 94 base pair length sequence of human β-globin desired to be amplified. The single base pair change which is associated with sickle cell anemia is depicted beneath the 94-mer.

FIG. **2** illustrates a photograph of an ethidium bromide-stained polyacrylamide gel demonstrating amplification of the 94-mer contained in human wild-type DNA and in a plasmid containing a 1.9 kb BamHI fragment of the normal β-globin gene (pBR328:HbA).

FIG. **3** illustrates a photograph of an ethidium bromide-stained polyacrylamide gel demonstrating amplification of any of the specific target 94-mer sequence present in pBR328:HbA, a plasmid containing a 1.9 kb BamHI fragment of the sickle cell allele of β-globin (pBR328:HbS), pBR328:HbA where the sequence to be amplified is cleaved with MstII, and pBR328:HbS where the sequence to be amplified has been treated but not cleaved with MstII.

FIG. **4** illustrates in detail the steps and products of the polymerase chain reaction for amplification of the desired 94-mer sequence of human β-globin for three cycles using two oligonucleotides primers.

FIG. **5** represents a photograph of an ethidium bromide-stained polyacrylamide gel demonstrating amplification after four cycles of a 240-mer sequence in pBR328:HbA, where the aliquots are digested with NcoI (Lane 3), MstII (Lane 4) or HinfI (Lane 5). Lane 1 is the molecular weight standard and Lane 2 contains the intact 240-bp product.

FIG. **6** illustrates the sequence of the normal (β^A) and sickle cell (β^S) β-globin genes in the region of the DdeI and HinfI restriction sites, where the single lines for β^A mark the position of the DdeI site (CTGAG) and the double bars for β^A and β^S mark the position of the HinfI site (GACTC).

FIG. **7** illustrates the results of sequential digestion of normal β-globin using a 40-mer probe and DdeI followed by HinfI restriction enzymes.

FIG. **8** illustrates the results of sequential digestion of sickle β-globin using the same 40-mer probe as in FIG. 7 and DdeI followed by HinfI restriction enzymes.

FIG. **9** illustrates a photograph of an ethidium bromide-stained polyacrylamide gel demonstrating the use of the same 40-mer probe as in FIG. 7 to specifically characterize the beta-globin alleles present in samples of whole human DNA which have been subjected to amplification, hybridization with the probe, and sequential digestion with DdeI and HinfI.

FIG. **10** illustrates a photograph of a 6% NuSieve agarose gel visualized using ethidium bromide and UV light. This photograph demonstrates amplification of a sub-fragment of a 110-bp amplification product which sub-fragment is an inner nested set within the 110-bp fragment.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "oligonucleotide" as used herein in referring to primers, probes, oligomer fragments to be detected, oligomer controls and unlabeled blocking oligomers is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three. Its exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide.

The term "primer" as used herein refers to an oligonucleotide whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase and at a suitable temperature and pH. The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature and source of primer. For example, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15–25 or more nucleotides, although it may contain fewer nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with template.

The primers herein are selected to be "substantially" complementary to the different strands of each specific sequence to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5′ end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to be am-

plified to hybridize therewith and thereby form a template for synthesis of the extension product of the other primer.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes each of which cut double-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "DNA polymorphism" refers to the condition in which two or more different nucleotide sequences coexist in the same interbreeding population in a DNA sequence.

The term "restriction fragment length polymorphism" ("RFLP") refers to the differences in DNA nucleotide sequences that are randomly distributed throughout the entire human genome and that produce different restriction endonuclease patterns.

The present invention is directed to a process for amplifying any one or more desired specific nucleic acid sequences suspected of being in a nucleic acid. Because large amounts of a specific sequence may be produced by this process, the present invention may be used for improving the efficiency of cloning DNA or messenger RNA and for amplifying a target sequence to facilitate detection thereof.

In general, the present process involves a chain reaction for producing, in exponential quantities relative to the number of reaction steps involved, at least one specific nucleic acid sequence given (a) that the ends of the required sequence are known in sufficient detail that oligonucleotides can be synthesized which will hybridize to them, and (b) that a small amount of the sequence is available to initiate the chain reaction. The product of the chain reaction will be a discrete nucleic acid duplex with termini corresponding to the ends of the specific primers employed.

Any source of nucleic acid, in purified or nonpurified form, can be utilized as the starting nucleic acid or acids, provided it is suspected of containing the specific nucleic acid sequence desired. Thus, the process may employ, for example, DNA or RNA, including messenger RNA, which DNA or RNA may be single stranded or double stranded. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of any of these nucleic acids may also be employed, or the nucleic acids produced from a previous amplification reaction herein using the same or different primers may be so utilized. The specific nucleic acid sequence to be amplified may be only a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor fraction of a complex mixture, such as a portion of the β-globin gene contained in whole human DNA or a portion of nucleic acid sequence due to a particular microorganism which organism might constitute only a very minor fraction of a particular biological sample. The starting nucleic acid may contain more than one desired specific nucleic acid sequence which may be the same or different. Therefore, the present process is useful not only for producing large amounts of one specific nucleic acid sequence, but also for amplifying simultaneously more than one different specific nucleic acid sequence located on the same or different nucleic acid molecules.

The nucleic acid or acids may be obtained from any source, for example, from plasmids such as pBR322, from cloned DNA or RNA, or from natural DNA or RNA from any source, including bacteria, yeast, viruses, and higher organisms such as plants or animals. DNA or RNA may be extracted from blood, tissue material such as chorionic villi or amniotic cells by a variety of techniques such as that described by Maniatis et al., *Molecular Cloning: A Laboratory Manual,* (New York: Cold Spring Harbor Laboratory, 1982), pp 280–281.

Any specific nucleic acid sequence can be produced by the present process. It is only necessary that a sufficient number of bases at both ends of the sequence be known in sufficient detail so that two oligonucleotide primers can be prepared which will hybridize to different strands of the desired sequence and at relative positions along the sequence such that an extension product synthesized from one primer, when it is separated from its template (complement), can serve as a template for extension of the other primer into a nucleic acid of defined length. The greater the knowledge about the bases at both ends of the sequence, the greater can be the specificity of the primers for the target nucleic acid sequence, and thus the greater the efficiency of the process. It will be understood that the word primer as used hereinafter may refer to more than one primer, particularly in the case where there is some ambiguity in the information regarding the terminal sequence(s) of the fragment to be amplified. For instance, in the case where a nucleic acid sequence is inferred from protein sequence information a collection of primers containing sequences representing all possible codon variations based on degeneracy of the genetic code will be used for each strand. One primer from this collection will be homologous with the end of the desired sequence to be amplified.

The oligonucleotide primers may be prepared using any suitable method, such as, for example, the phosphotriester and phosphodiester methods described above, or automated embodiments thereof. In one such automated embodiment diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage et al., *Tetrahedron Letters* (1981), 22:1859–1862. One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066. It is also possible to use a primer which has been isolated from a biological source (such as a restriction endonuclease digest).

The specific nucleic acid sequence is produced by using the nucleic acid containing that sequence as a template. If the nucleic acid contains two strands, it is necessary to separate the strands of the nucleic acid before it can be used as the template, either as a separate step or simultaneously with the synthesis of the primer extension products. This strand separation can be accomplished by any suitable denaturing method including physical, chemical or enzymatic means. One physical method of separating the strands of the nucleic acid involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation may involve temperature ranging from about 80° to 105° C. for times ranging from about 1 to 10 minutes. Strand separation may also be induced by an enzyme from the class of enzymes known as helicases or the enzyme RecA, which has helicase activity and in the presence of riboATP is known to denature DNA. The reaction conditions suitable for separating the strands of nucleic acids with helicases are described by *Cold Spring Harbor Symposia on Quantitative Biology,* Vol. XLIII "DNA: Replication and Recombination" (New York:

Cold Spring Harbor Laboratory, 1978), B. Kuhn et al., "DNA Helicases", pp. 63–67, and techniques for using RecA are reviewed in C. Radding, *Ann. Rev. Genetics*, 16:405–37 (1982).

If the original nucleic acid containing the sequence to be amplified is single stranded, its complement is synthesized by adding one or two oligonucleotide primers thereto. If an appropriate single primer is added, a primer extension product is synthesized in the presence of the primer, an agent for polymerization and the four nucleotides described below. The product will be partially complementary to the single-stranded nucleic acid and will hybridize with the nucleic acid strand to form a duplex of unequal length strands that may then be separated into single strands as described above to produce two single separated complementary strands. Alternatively, two appropriate primers may be added to the single-stranded nucleic acid and the reaction carried out.

If the original nucleic acid constitutes the sequence to be amplified, the primer extension product(s) produced will be completely complementary to the strands of the original nucleic acid and will hybridize therewith to form a duplex of equal length strands to be separated into single-stranded molecules.

When the complementary strands of the nucleic acid or acids are separated, whether the nucleic acid was originally double or single stranded, the strands are ready to be used as a template for the synthesis of additional nucleic acid strands. This synthesis can be performed using any suitable method. Generally it occurs in a buffered aqueous solution, preferably at a pH of 7–9, most preferably about 8. Preferably, a molar excess (for cloned nucleic acid, usually about 1000:1 primer:template, and for genomic nucleic acid, usually about 10^6: 1 primer:template) of the two oligonucleotide primers is added to the buffer containing the separated template strands. It is understood, however, that the amount of complementary strand may not be known if the process herein is used for diagnostic applications, so that the amount of primer relative to the amount of complementary strand cannot be determined with certainty. As a practical matter, however, the amount of primer added will generally be in molar excess over the amount of complementary strand (template) when the sequence to be amplified is contained in a mixture of complicated long-chain nucleic acid strands. A large molar excess is preferred to improve the efficiency of the process.

The deoxyribonucleoside triphosphates dATP, dCTP, dGTP and TTP are also added to the synthesis mixture in adequate amounts and the resulting solution is heated to about 90°–100° C. for from about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period the solution is allowed to cool to from 20°–40° C., which is preferable for the primer hybridization. To the cooled mixture is added an agent for polymerization, and the reaction is allowed to occur under conditions known in the art. This synthesis reaction may occur at from room temperature up to a temperature above which the agent for polymerization no longer functions efficiently. Thus, for example, if DNA polymerase is used as the agent for polymerization, the temperature is generally no greater than about 45° C. Preferably an amount of dimethylsulfoxide (DMSO) is present which is effective in detection of the signal or the temperature is 35°–40° C. Most preferably, 5–10% by volume DMSO is present and the temperature is 35°–40° C. For certain applications, where the sequences to be amplified are over 110 base pair fragments, such as the HLA DQ-α or -β genes, an effective amount (e.g., 10% by volume) of DMSO is added to the amplification mixture, and the reaction is carried at 35°–40° C., to obtain detectable results or to enable cloning.

The agent for polymerization may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase, and other enzymes, including heatstable enzymes, which will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths. There may be agents, however, which initiate synthesis at the 5' end and proceed in the other direction, using the same process as described above.

The newly synthesized strand and its complementary nucleic acid strand form a double-stranded molecule which is used in the succeeding steps of the process. In the next step, the strands of the double-stranded molecule are separated using any of the procedures described above to provide single-stranded molecules.

New nucleic acid is synthesized on the single-stranded molecules. Additional inducing agent, nucleotides and primers may be added if necessary for the reaction to proceed under the conditions prescribed above. Again, the synthesis will be initiated at one end of the oligonucleotide primers and will proceed along the single strands of the template to produce additional nucleic acid. After this step, half of the extension product will consist of the specific nucleic acid sequence bounded by the two primers.

The steps of strand separation and extension product synthesis can be repeated as often as needed to produce the desired quantity of the specific nucleic acid sequence. As will be described in further detail below, the amount of the specific nucleic acid sequence produced will accumulate in an exponential fashion.

When it is desired to produce more than one specific nucleic acid sequence from the first nucleic acid or mixture of nucleic acids, the appropriate number of different oligonucleotide primers are utilized. For example, if two different specific nucleic acid sequences are to be produced, four primers are utilized. Two of the primers are specific for one of the specific nucleic acid sequences and the other two primers are specific for the second specific nucleic acid sequence. In this manner, each of the two different specific sequences can be produced exponentially by the present process.

The present invention can be peformed in a step-wise fashion where after each step new reagents are added, or simultaneously, where all reagents are added at the initial step, or partially step-wise and partially simultaneous, where fresh reagent is added after a given number of steps. If a method of strand separation, such as heat, is employed which will inactivate the agent for polymerization, as in the case of a heat-labile enzyme, then it is necessary to replenish the agent for polymerization after every strand separation step. The simultaneous method may be utilized when a number of puri-

fied components, including an enzymatic means such as helicase, is used for the strand separation step. In the simultaneous procedure, the reaction mixture may contain, in addition to the nucleic acid strand(s) containing the desired sequence, the strand-separating enzyme (e.g., helicase), an appropriate energy source for the strand-separating enzyme, such as rATP, the four nucleotides, the oligonucleotide primers in molar excess, and the inducing agent, e.g., Klenow fragment of *E. coli* DNA polymerase I. If heat is used for denaturation in a simultaneous process, a heat-stable inducing agent such as a thermostable polymerase may be employed which will operate at an elevated temperature, preferably 65°-90° C. depending on the inducing agent, at which temperature the nucleic acid will consist of single and double strands in equilibrium. For smaller lengths of nucleic acid, lower temperatures of about 50° C. may be employed. The upper temperature will depend on the temperature at which the enzyme will degrade or the temperature above which an insufficient level of primer hybridization will occur. Such a heat-stable enzyme is described, e.g., by A. S. Kaledin et al., *Biokhimiya*, 45, 644-651 (1980). Each step of the process will occur sequentially notwithstanding the initial presence of all the reagents. Additional materials may be added as necessary. After the appropriate length of time has passed to produce the desired amount of the specific nucleic acid sequence, the reaction may be halted by inactivating the enzymes in any known manner or separating the components of the reaction.

The process of the present invention may be conducted continuously. In one embodiment of an automated process, the reaction may be cycled through a denaturing region, a reagent addition region, and a reaction region. In another embodiment, the enzyme used for the synthesis of primer extension products can be immobilized in a column. The other reaction components can be continuously circulated by a pump through the column and a heating coil in series; thus the nucleic acids produced can be repeatedly denatured without inactivating the enzyme.

The present invention is demonstrated diagrammatically below where double-stranded DNA containing the desired sequence [S] comprised of complementary strands [S+] and [S−] is utilized as the nucleic acid. During the first and each subsequent reaction cycle extension of each oligonucleotide primer on the original template will produce one new ssDNA molecule product of indefinite length which terminates with only one of the primers. These products, hereafter referred to as "long products," will accumulate in a linear fashion; that is, the amount present after any number of cycles will be proportional to the number of cycles.

The long products thus produced will act as templates for one or the other of the oligonucleotide primers during subsequent cycles and will produce molecules of the desired sequence [S+] or [S−]. These molecules will also function as templates for one or the other of the oligonucleotide primers, producing further [S+] and [S−], and thus a chain reaction can be sustained which will result in the accumulation of [S] at an exponential rate relative to the number of cycles.

By-products formed by oligonucleotide hybridizations other than those intended are not self-catalytic (except in rare instances) and thus accumulate at a linear rate.

The specific sequence to be amplified, [S], can be depicted diagrammatically as:

[S+] 5′ AAAAAAAAAAXXXXXXXXXXCCCCCCCCCC 3′
[S−] 5′ TTTTTTTTTTYYYYYYYYYYGGGGGGGGGG 5′

The appropriate oligonucleotide primers would be:

Primer 1: GGGGGGGGGG
Primer 2: AAAAAAAAAA

so that if DNA containing [S]

. . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
. . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . .

is separated into single strands and its single strands are hybridized to Primers 1 and 2, the following extension reactions can be catalyzed by DNA polymerase in the presence of the four deoxyribonucleoside triphosphates:

3′ 5′
extends ← GGGGGGGGGG Primer 1
. . . zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
original template strand+

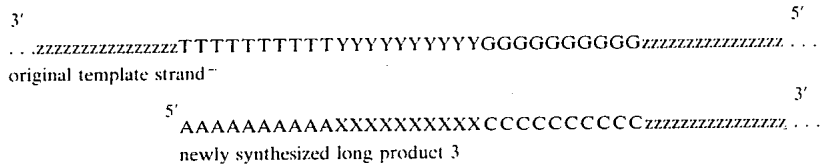
original template strand−
. . . zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGGzzzzzzzzzzzzzzzz . . .
Primer 2 AAAAAAAAAA → extends
5′ 3′

On denaturation of the two duplexes formed, the products are:

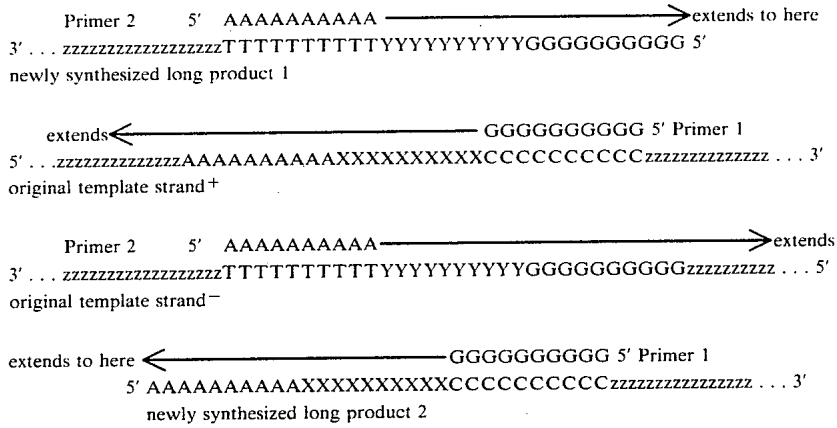
3′ 5′
. . .zzzzzzzzzzzzzzzzTTTTTTTTTTYYYYYYYYYYGGGGGGGGGG
newly synthesized long product 1

5′ 3′
. . .zzzzzzzzzzzzzzzzAAAAAAAAAAXXXXXXXXXXCCCCCCCCCCzzzzzzzzzzzzzzzz . . .
original template strand+

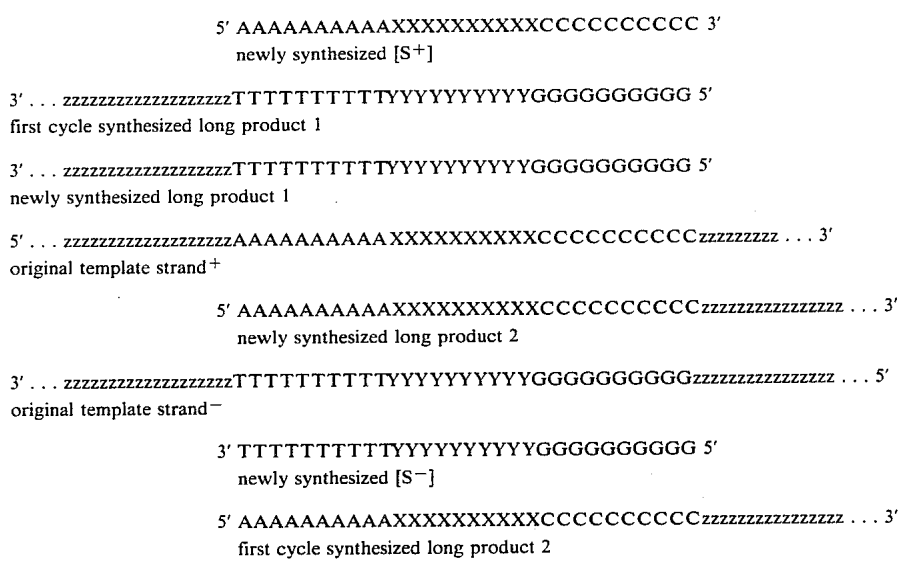
-continued



If these four strands are allowed to rehybridize with Primers 1 and 2 in the next cycle, agent for polymerization will catalyze the following reactions:



If the strands of the above four duplexes are separated, the following strands are found:



It is seen that each strand which terminates with the oligonucleotide sequence of one primer and the complementary sequence of the other is the specific nucleic acid sequence [S] that is desired to be produced.

The steps of this process can be repeated indefinitely, being limited only by the amount of Primers 1 and 2, the agent for polymerization and nucleotides present. For detection, the number of cycles used is that required to produce a detectable signal, an amount which will depend, e.g., on the nature of the sample. For example, if the sample is pure or diluted, fewer cycles may be required than if it is a complex mixture. If the sample is human genomic DNA, preferably the number of cycles is from about 10–30.

The amount of original nucleic acid remains constant in the entire process, because it it not replicated. The amount of the long products increases linearly because they are produced only from the original nucleic acid. The amount of the specific sequence increases exponentially. Thus, the specific sequence will become the predominant species. This is illustrated in the following table, which indicates the relative amounts of the species theoretically present after n cycles, assuming 100% efficiency at each cycle:

	Number of Double Strands After 0 to n Cycles		
Cycle Number	Template	Long Products	Specific Sequence [S]
0	1	—	—

-continued

Cycle Number	Number of Double Strands After 0 to n Cycles: Template	Long Products	Specific Sequence [S]
1	1	1	0
2	1	2	1
3	1	3	4
5	1	5	26
10	1	10	1013
15	1	15	32,752
20	1	20	1,048,555
n	1	n	$(2^n - n - 1)$

When a single-stranded nucleic acid is utilized as the template, only one long product is formed per cycle.

The method herein may be utilized to clone a particular nucleic acid sequence for insertion into a suitable expression vector. The vector may then be used to transform an appropriate host organism to produce the gene product of the sequence by standard method of recombinant DNA technology.

Normally, such cloning would either involve direct ligation into a vector or the addition of oligonucleotide linkers followed by restriction enzyme cleavage. Both of these methods involve, however, the inefficient blunt-end ligation reaction. Also, neither technique would control for the orientation or multiplicity of insertion of the amplified product into the cloning vector.

The amplification process herein may yield a mixture of nucleic acids, resulting from the original template nucleic acid, the expected target amplified products, and various background non-target products. The amplified product can also be a mixture if the original template DNA contains multiple target sequences, such as in a heterozygous diploid genome or when there is a family of related genes.

The primers herein may be modified to assist the rapid and specific cloning of the mixture of DNAs produced by the amplification reaction. In such modification the same or different restriction sites are incorporated at the 5′ ends of the primers to result in restriction sites at the two ends of the amplified product. When cut with the appropriate enzymes, the amplified product can then be easily inserted into plasmid or viral vectors and cloned. This cloning allows the analysis or expression of individual amplified products, not a mixture.

Although the same restriction site can be used for both primers, the use of different sites allows the insertion of the product into the vector with a specific orientation and suppresses multiple insertions as well as insertions arising from amplifications based on only one of the two primers. The specific orientation is useful when cloning into single-strand sequencing vectors, when single-strand hybridization probes are used, or when the cloned product is being expressed.

One method to prepare the primers is to choose a primer sequence which differs minimally from the target sequence. Regions in which each of the primers is to be located are screened for homology to restriction sites appropriate to the desired vector. For example, the target sequence "CAGTATCCGA . . . " differs by only one base from one containing a BamHI site. A primer sequence is chosen to match the target exactly at its 3′ end, and to contain the altered sequence and restriction site near its 5′ end (for example, "CAGgATCCGA . . . ", where the lower case letter symbolizes a mismatch with the target sequence). This minimally altered sequence will not interfere with the ability of the primer to hybridize to the original target sequence and to initiate polymerization. After the first amplification cycle the primer is copied, becomes the target, and matches exactly with new primers. After the amplification process, the products are cleaved with the appropriate restriction enzymes, optionally separated from inhibitors of ligation such as the nucleotide triphosphates and salts by passing over a desalting column or molecular weight chromatography column, and inserted by ligation into a cloning vector such as bacteriophage M13. The gene may then be sequenced and/or expressed using well known techniques.

The second method for preparing the primers involves taking the 3′ end of the primers from the target sequence and adding the desired restriction site(s) to the 5′ end of the primer. For the above example, a HindIII site could be added to make the sequence "cgaagctt-CAGTATCCGA . . . ", where lower case letters are as described above. The added bases would not contribute to the hybridization in the first cycle of amplification, but would match in subsequent cycles. The final amplified products are then cut with restriction enzyme(s) and cloned and expressed as described above. The gene being amplified may be, for example, human beta-hemoglobin or the human HLA DQ, DR or DP-α and -β genes.

In addition, the process herein can be used for in vitro mutagenesis. The oligodeoxyribonucleotide primers need not be exactly complementary to the DNA sequence which is being amplified. It is only necessary that they be able to hybridize to the sequence sufficiently well to be extended by the polymerase enzyme or by whatever other inducing agent is employed. The product of a polymerase chain reaction wherein the primers employed are not exactly complementary to the original template will contain the sequence of the primer rather than the template, thereby introducing as in vitro mutation. In further cycles this mutation will be amplified with an undiminished efficiency because no further mispaired primings are required. The mutant thus produced may be inserted into an appropriate vector by standard molecular biological techniques and might confer mutant properties on this vector such as the potential for production of an altered protein.

The process of making an altered DNA sequence as described above could be repeated on the altered DNA using different primers so as to induce further sequence changes. In this way a series of mutated sequences could gradually be produced wherein each new addition to the series could differ from the last in a minor way, but from the original DNA source sequence in an increasingly major way. In this manner changes could be made ultimately which were not feasible in a single step due to the inability of a very seriously mismatched primer to function.

In addition, the primer can contain as part of its sequence a non-complementary sequence provided that a sufficient amount of the primer contains a sequence which is complementary to the strand to be amplified. For example, a nucleotide sequence which is not complementary to the template sequence (such as, e.g., a promoter, linker, coding sequence, etc.) may be attached at the 5′ end of one or both of the primers, and thereby appended to the product of the amplification process. After the extension primer is added, sufficient cycles are run to achieve the desired amount of new

template containing the non-complementary nucleotide insert. This allows production of large quantities of the combined fragments in a relatively short period of time (e.g., two hours or less) using a simple technique.

Moreover, the process herein may be used to synthesize a nucleic acid fragment from an existing nucleic acid fragment which is shorter than its product (called the core segment) using certain primers the 3′ ends of which are complementary to or substantially complementary to the 3′ ends of the single strands produced by separating the strands of the original shorter nucleic acid fragments, and the 5′ ends of which primers contain sequence information to be appended to the core segment. This process comprises:

(a) treating the strands of said existing fragment with two oligonucleotide primers under conditions such that an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primers are selected so as to be substantially complementary to the 3′ end of each strand of said existing fragment such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein each primer contains, at its 5′ end, a sequence of nucleotides which are not complementary to said existing fragment and which correspond to the two ends of the nucleic acid fragment being synthesized;

(b) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

(c) treating tthe single-stranded molecules generated from step (b) with the primers of step (a) under conditions such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template so as to produce two intermediate double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ end of one of the oligonucleotide primers, and two full-length double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ ends of both of the oligonucleotide primers;

(d) repeating steps (b) and (c) for a sufficient number of times to produce the full-length double-stranded molecule in an effective amount;

(e) treating the strands of the product of step (d) with two primers so as to lengthen the product of step (d) on both ends; and

(f) repeating steps (a)–(d) using the product of step (d) as the core fragment and two oligonucleotide primers which are complementary or substantially complementary to the 3′ ends of the single strands produced by separating the strands of the product of step (d).

Steps (b) and (c) are repeated as often as necessary, usually at least 5 times, to produce the required amount of the full-length double-stranded product to synthesize the final product (i.e., the effective amount). In addition, the core segment may be obtained as the product of a previous amplification cycle. The product produced in step (d) may be purified before a new cycle of extension and amplification, or used directly by employing the reaction mixture containing the product.

If the 3′ ends of the primers are not exactly complementary to the 3′ ends of the single strands of the original shorter nucleic acid, the core fragment of the product will not be exactly the same as the sequence information resident in the original shorter nucleic acid. Therefore, mutants of the original nucleic acid may be made by using primers which are substantially complementary at their 3′ ends to the 3′ ends of the single strands of the original shorter nucleic acid.

If restriction site linkers are incorporated into the primers, then the amplified double-stranded products can be digested with the appropriate restriction enzymes and ligated directly into an M13 vector for rapid cloning and sequencing. The M13 plaques containing the specific amplified target sequences can be identified by hybridizing plaque lift filters with a probe specific for the target sequence.

The method herein may also be used to enable detection and/or characterization of specific nucleic acid sequences associated with infectious diseases, genetic disorders or cellular disorders such as cancer, e.g., oncogenes. Amplification is useful when the amount of nucleic acid available for analysis is very small, as, for example, in the prenatal diagnosis of sickle cell anemia using DNA obtained from fetal cells. Amplification is particularly useful if such an analysis is to be done on a small sample using non-radioactive detection techniques which may be inherently insensitive, or where radioactive techniques are being employed but where rapid detection is desirble.

For purposes of this invention genetic diseases may include specific deletions and/or mutations in genomic DNA from any organism, such as, e.g., sickle cell anemia, cystic fibrosis, α-thalassemia, β-thalassemia, and the like. Sickle cell anemia can be readily detected via oligomer restriction analysis or a RFLP-like analysis following amplification of the appropriate DNA sequence by the present method. α-Thalassemia can be detected by the absence of a sequence, and β-thalassemia can be detected by the presence of a polymorphic restriction site closely linked to a mutation which causes the disease.

All of these genetic diseases may be detected by amplifying the appropriate sequence and analyzing it by Southern blots without using radioactive probes. In such a process, for example, a small sample of DNA from, e.g., amniotic fluid containing a very low level of the desired sequence is amplified, cut with a restriction enzyme, and analyzed via a Southern blotting technique. The use of non-radioactive probes is facilitated by the high level of the amplified signal.

In another embodiment a small sample of DNA may be amplified to a convenient level and then a further cycle of extension reactions performed wherein nucleotide derivatives which are readily detectable (such as ^{32}P-labeled or biotin labeled nucleoside triphosphates) are incorporated directly into the final DNA product, which may be analyzed by restriction and electrophoretic separation or any other appropriate method. An example of this technique in a model system is demonstrated in FIG. 5.

In a further embodiment, demonstrated in a model system in FIG. 3, the nucleic acid may be exposed to a particular restriction endonuclease prior to amplification. Since a sequence which has been cut cannot be amplified, the appearance of an amplified fragment, despite prior restriction of the DNA sample, implies the absence of a site for the endonuclease within the amplified sequence. The presence or absence of an amplified sequence can be detected by an appropriate method.

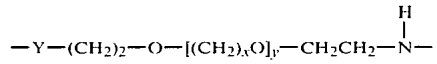
A practical application of this technique can be illustrated by its use in facilitating the detection of sickle cell anemia via the oligomer restriction technique described

herein and in copending U.S. application Ser. No. 716,982 filed Mar. 27, 1985. Sickle cell anemia is a hemoglobin disease which is caused by a single base pair change in the sixth codon of the β-globin gene. FIG. 6 illustrates the sequences of normal and sickle cell β-globin genes in the region of their polymorphism, where the single bars mark the location of a DdeI site present only in the normal gene and where the double bars mark the location of a HinfI site which is non-polymorphic and thus present in both the normal and sickle cell alleles. FIG. 7 illustrates the process of oligomer restriction of normal β-globin DNA using a probe spanning both restriction sites and labeled where the asterisk appears. (The probe is preferably labeled at the end which is fewer base pairs from the restriction site than the other end of the probe.) The DNA, amplified as provided herein, is denatured and annealed to the labeled probe. The amplification may be carried out at elevated temperatures (35°–40° C.) in the presence of dimethyl sulfoxide to minimize formation of secondary structure. The enzyme DdeI cleaves the DNA at the reformed DdeI site and generates a labeled octamer. Under the conditions used in the test the octamer is short enough to dissociate from the duplex. The subsequent addition of the enzyme HinfI has no effect on the now single-stranded octamer. FIG. 8 illustrates the same process applied to the sickle cell allele of β-globin DNA. The enzyme DdeI cannot cleave the duplex formed by the amplified DNA and the labeled probe because of the A-A base pair mismatch. The enzyme HinfI, however, does restrict the hybrid and a labeled trimer is produced. In practice the method can diagnose the DNA of an individual as being either homozygous for the wild type, homozygous for the sickle type or a heterozygous carrier of the sickle cell trait, since a specific signal is associated with the presence of eithr allele. Use of this above-described method to amplify the pertinent sequence allows for a rapid analysis of a single copy gene using a probe with only a single ^{32}P label.

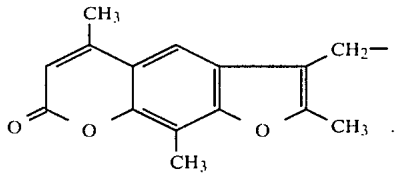
Various infectious diseases can be diagnosed by the presence in clinical samples of specific DNA sequences characteristic of the causative microorganism. These include bacteria, such as Salmonella, Chlamydia, and Neisseria; viruses, such as the hepatitis viruses; and parasites, such as the Plasmodium responsible for malaria. U.S. Pat. No. 4,358,535 issued to Falkow describes the use of specific DNA hybridization probes for the diagnosis of infectious diseases. A problem inherent in the Falkow procedure is that a relatively small number of pathogenic organisms may be present in a clinical sample from an infected patient and the DNA extracted from these may constitute only a very small fraction of the total DNA in the sample. Specific amplification of suspected sequences prior to immobilization and hybridization detection of the DNA samples could greatly improve the sensitivity and specificity of these procedures.

Routine clinical use of DNA probes for the diagnosis of infectious diseases would be simplified considerably if non-radioactively labeled probes could be employed as described in EP No. 63,879 to Ward. In this procedure biotin-containing DNA probes are detected by chromogenic enzymes linked to avidin or biotin-specific antibodies. This type of detection is convenient, but relatively insensitive. The combination of specific DNA amplification by the present method and the use of stably labeled probes could provide the convenience and sensitivity required to make the Falkow and Ward procedures useful in a routine clinical setting.

In addition, the probe may be a biotinylated probe in which the biotin is attached to a spacer arm of the formula:



where Y is O, NH or N—CHO, x is a number from 1 to 4, and y is a number from 2 to 4. The spacer arm is in turn attached to a psoralen moiety of the formula:



The psoralen moiety intercalates into and crosslinks a "gapped circle" probe as described by Courage-Tebbe et al., *Biochim. Biophys. Acta*, 697 (1982) 1–5, wherein the single-stranded hybridization region of the gapped circle spans the region contained in the primers. The details of this biotinylation and dot blot procedure are described more fully in commonly assigned copending U.S. application Ser. Nos. 683,263 filed Dec. 18, 1984 and 791,332 filed Oct. 25, 1985, the disclosures of which are incorporated herein by reference.

The amplification process can also be utilized to produce sufficient quantities of DNA from a single copy human gene such that detection by a simple non-specific DNA stain such as ethidium bromide can be employed so as to make a DNA diagnosis directly.

In addition to detecting infectious diseases and pathological abnormalities in the genome of organisms, the process herein can also be used to detect DNA polymorphism which may not be associated with any pathological state.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner. In these examples all percentages are by weight if for solids and by volume if for liquids, and all temperatures are in degrees Celsius unless otherwise noted.

EXAMPLE 1

A 25 base pair sequence having the nucleotide sequence

5′ CCTCGGCACCGTCACCCTGGATGCT 3′

3′ GGAGCCGTGGCAGTGGGACCTACGA 5′

contained on a 47 base pair FokI restriction fragment of pBR322 obtainable from ATCC was prepared as follows. A FokI digest of pBR322 containing the 47-bp fragment was produced by digesting pBR322 with FokI in accordance with the conditions suggested by the supplier, New England Biolabs Inc. The primers which were utilized were 5′ d(CCTCGGCACCG) 3′ and 5′ d(AGCATCCAGGGTG) 3′, and were prepared using conventional techniques. The following ingredients were added to 33 μl of buffer which consisted of 25 mM potassium phosphate, 10 mM magnesium chloride and 100 mM sodium chloride at pH 7.5: 2433 pmoles of each of the primers described above, 2.4 pmoles of the FokI digest of pBR322, 12 nmoles of dATP, 22 nmoles of dCTP, 19 nmoles of dGTP and 10 nmoles of TTP.

The mixture was heated to 85° C. for five minutes and allowed to cool to ambient temperature. Five units of the Klenow fragment of *E. coli* DNA polymerase I were added and the temperature was maintained for 15 minutes. After that time, the mixture was again heated to 85° C. for five minutes and allowed to cool. Five units of the Klenow fragment were again added and the reaction was carried out for 15 minutes. The heating, cooling and synthesis steps were repeated eleven more times.

After the final repetition, a 5 μl aliquot was removed from the reaction mixture. This was heated to 85° C. for three minutes and allowed to cool to ambient temperature. 12.5 pmoles of α-P^{32}-deoxycytidine triphosphate and 5 units of Klenow fragment were added and the reaction was allowed to proceed for 15 minutes. The labeled products were examined by polyacrylamide gel electrophoresis. The FokI digest was labeled in a similar fashion and served as a control and molecular weight markers. The only heavily labeled band visible after the 13 cycles was the intended 25 base pair sequence.

EXAMPLE 2

The desired sequence to be amplified was a 94 base pair sequence contained within the human beta-globin gene and spanning the MstII site involved in sickle cell anemia. The sequence has the nucleotide sequence shown in FIG. 1.

I. Synthesis of Primers

The following two oligodeoxyribonucleotide primers were prepared by the method described below:

5′ CACAGGGCAGTAACG 3′ Primer A

and

5′ TTTGCTTCTGACACA 3′ Primer B

Automated Synthesis Procedures: The diethylphosphoramidites, synthesized according to Beaucage and Caruthers (*Tetrahedron Letters* (1981) 22:1859–1862), were sequentially condensed to a nucleotide derivatized controlled pore glass support using a Biosearch SAM-1. The procedure included detritylation with trichloroacetic acid in dichlormethane, condensation using benzotriazole as activating proton donor, and capping with acetic anhydride and dimethylaminopyridine in tetrahydrofuran and pyridine. Cycle time was approximately 30 minutes. Yields at each step were essentially quantitative and were determined by collection and spectroscopic examination of the dimethoxytrityl alcohol released during detritylation.

Oligodeoxyribonucleotide Deprotection and Purification Procedures: The solid support was removed from the column and exposed to 1 ml concentrated ammonium hydroxide at room temperature for four hours in a closed tube. The support was then removed by filtration and the solution containing the partially protected oligodeoxyribonucleotide was brought to 55° C. for five hours. Ammonia was removed and the residue was applied to a preparative polyacrylamide gel. Electrophoresis was carried out at 30 volts/cm for 90 minutes after which the band containing the product was identified by UV shadowing of a fluorescent plate. The band was excised and eluted with 1 ml distilled water overnight at 4° C. This solution was applied to an Altech RP18 column and eluted with a 7–13% gradient of acetonitrile in 1% ammonium acetate buffer at pH 6.0. The elution was monitored by UV absorbance at 260 nm and the appropriate fraction collected, quantitated by UV absorbance in a fixed volume and evaporated to dryness at room temperature in a vacuum centrifuge.

Characterization of Oligodeoxyribonucleotides: Test aliquots of the purified oligonucleotides were ^{32}P labeled with polynucleotide kinase and γ-^{32}P-ATP. The labeled compounds were examined by autoradiography of 14–20% polyacrylamide gels after electrophoresis for 45 minutes at 50 volts/cm. This procedure verifies the molecular weight. Base composition was determined by digestion of the oligodeoxyribonucleotide to nucleosides by use of venom diesterase and bacterial alkaline phosphatase and subsequent separation and quantitation of the derived nucleosides using a reverse phase HPLC column and a 10% acetonitrile, 1% ammonium acetate mobile phase.

II. Source of DNA

A. Extraction of Whole Human Wild-Type DNA

Human geonomic DNA homozygous for normal β-globin was extracted from the cell line Molt4 (obtained from Human Genetic Mutant Cell Repository and identified as GM2219c) using the technique described by Stetler et al., *Proc. Nat. Acad. Sci.* USA (1982), 79:5966–5970.

B. Construction of Cloned Globin Genes

A 1.9 kb BamHI fragment of the normal β-globin gene was isolated from the cosmid pFC11 and inserted into the BamHI site of pBR328 (Soberon, et al., Gene (1980) 9:287–305). This fragment, which encompasses the region that hybridizes to the synthetic 40-mer probe, includes the first and second exons, first intron, and 5′ flanking sequences of the gene (Lawn et al., *Cell* (1978), 15:1157–1174). This clone was designated pBR328:HbA and deposited under ATCC No. 39,698 on May 25, 1984.

The corresponding 1.9 kb BamHI fragment of the sickle cell allele of β-globin was isolated from the cosmid pFC12 and cloned as described above. This clone was designated pBR328:HbS and deposited under ATCC No. 39,699 on May 25, 1984.

Each recombinant plasmid was transformed into and propagated in *E. coli* MM294 (ATCC No. 39,607).

C. Digestion of Cloned Globin Genes with MstII

A total of 100 μg each of pBR328:HbA and pBR328:HbS were individually digested with 20 units of MstII (New England Biolabs) for 16 hours at 37° C. in 200 μl of 150 mM NaCl, 12 mM Tris HCl (pH 7.5), 12 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 100 μg/ml bovine serum albumin (BSA). The products are designated pBR328:HbA/MstII and pBR328:HbS/MstII, respectively.

III. Polymerase Chain Reaction

To 100 μl of buffer consisting of 60 mM sodium acetate, 30 mM Tris acetate and 10 mM magnesium acetate at pH 8.0 was added 2 μl of a solution containing 100 picomoles of Primer A (of the sequence d(CACAGGGCACTAACG)). 100 picomoles of Primer B (of the sequence d(TTTGCTTCTGACACA)) and 1000 picomoles each of dATP, dCTP, dGTP and TTP. In addition, one of the following sources of DNA described above was added:

10 μg whole human wild-type DNA (Reaction I)

0.1 picomole pBR328:HbA (Reaction II)

0.1 picomole pBR328:HbS (Reaction III)

0.1 picomole pBR328:HbA/MstII (Reaction IV)
0.1 picomole pBR328:HbS/MstII (Reaction V)
No target DNA (Reaction VI)

Each resulting solution was heated to 100° C. for four minutes and allowed to cool to room temperature for two minutes, whereupon 1 μl containing four units of Klenow fragment of *E. coli* DNA polymerase was added. Each reaction was allowed to proceed for 10 minutes, after which the cycle of adding the primers, nucleotides and DNA, heating, cooling, adding polymerase, and reacting was repeated nineteen times for Reaction I and four times for Reactions II–VI.

Four microliter aliquots of Reactions I and II removed before the first cycle and after the last cycle of each reaction were applied to a 12% polyacrylamide gel 0.089M in Tris-borate buffer at pH 8.3 and 2.5 mM in EDTA. The gel was electrophoresed at 25 volts/cm for four hours, transferred to a nylon membrane serving as solid phase support and probed with a 5'-^{32}P-labeled 40 pb synthetic fragment, prepared by standared techniques, of the sequence

5'd(TCCTGAGGAGAAGTCTGCCGT-
TACTGCCCTGTGGGGCAAG)3'

in 30% formamide, 3×SSPE, 5×Denhardt's, 5% sodium dodecyl sulfate at pH 7.4. FIG. 2 is an autoradiograph of the probed nylon membrane for Reactions I and II. Lane 1 is 0.1 picomole of a 58-bp synthetic fragment control one strand of which is complementary to the above probe. Lane 2 is 4 μl of Reaction I prior to the first amplification cycle. Lane 3 is 4 μl of Reaction I after the 20th amplification cycle. Lane 4 is 4 μl of Reaction II after five amplification cycles. Lane 5 is a molecular weight standard consisting of a FokI (New England Biolabs) digest of pBR322 (New England Biolabs) labeled with alpha-^{32}P-dNTPs and polymerase. Lane 3 shows that after twenty cycles the reaction mixture I contained a large amount of the specific sequence of the proper molecular weight and no other detectable products. Reaction mixture II after five cycles also contained this product, as well as the starting nucleic acid and other products, as shown by Lane 4.

To 5.0 μl aliquots of Reactions II–VI after the fourth cycle were added 5 pmoles of each primer described above. The solutions were heated to 100° C. for four minutes and allowed to equilibrate to room temperature. Three pmoles each of alpha-^{32}P-dATP, alpha-^{32}P-dCTP, alpha-^{32}P-dGTP and alpha-^{32}P-TTP and four units of Klenow fragment were added. The reaction, in a final volume of 10 μl and at the salt concentrations given above, was allowed to proceed for 10 minutes. The polymerase activity was terminated by heating for 20 minutes at 60° C. Four μl aliquots of Reactions II–VI were loaded onto a 12% polyacrylamide gel 0.089M in Tris-borate buffer at pH 8.3 and 2.5 mM in EDTA. The gel was electrophoresed at 25 volts/cm for four hours after which autoradiography was performed.

FIG. 3 is an autoradiograph of the electrophoresis. Lane 1 is a molecular weight standard, Lane 2 is Reaction II, Lane 3 is Reaction III, Lane 4 is Reaction IV and Lane 5 is Reaction V. Another lane for Reaction VI with no DNA as control had no images in any of the lanes. It can be seen from the figure that the 94-bp fragment predicted from the target DNA was present only where intact β-globin DNA sequences were available for amplification, i.e., pBR328:HbA (Lane 2), pBR328:HbS (Lane 3) and pBR328:HbS(MstII (Lane 5). MstII digestion cuts pBR328:HbA in the 94-mer sequence rendering it incapable of being amplified, and the 94-mer band does not appear in Lane 4. In contrast, the 94-mer sequence in pBR328:HbS does not cut when the plasmid is digested with MstII and thus is available for amplification as shown in Lane 5.

FIG. 4 illustrates the chain reaction for three cycles in amplifying the 94-bp sequence. PC01 and PC02 are Primers A and B. The numbers on the right indicate the cycles, whereas the numbers on the left indicate the cycle number in which a particular molecule was produced.

EXAMPLE 3

This example illustrates amplification of a 110 bp sequence spanning the allelic MstII site in the human hemoglobin gene.

A total of 1.0 microgram whole human DNA, 100 picomoles d(ACACAACTGTGTTCACTAGC) and 100 picomoles d(CAACTTCATCCACGTTCACC), the primers having been prepared by the technique of Example 2, were dissolved in 100 μl of a solution which was:

1.5 mM in each of the four deoxyribonucleoside triphosphates
30 mM in Tris acetate buffer at pH 7.9
60 mM in sodium acetate
10 mM in magnesium acetate
0.25 mM in dithiothreitol

The solution was heated to 100° C. for one minute and brought rapidly to 25° C. for one minute, after which was added 2.5 units Klenow fragment of DNA polymerase. The polymerase reaction was allowed to proceed for two minutes at 25° C., after which the cycle of heating, cooling, adding Klenow, and reacting was repeated as often as desired.

With 70% efficiency at each cycle, 15 cycles resulted in the synthesis of 1.4 femtomoles of the desired 110 bp fragment of the β-globin gene.

EXAMPLE 4

This example illustrates amplification of a 240 bp sequence spanning the allelic MstII site in the human hemoglobin gene. This sequence contains NcoI, HinfI and MstII restriction sites.

To 100 μl of a mixture of 60 mM sodium acetate, 30 mM Tris acetate and 10 mM magnesium acetate at pH 8.0 containing 0.1 pmole pBR328:HbA was added 2 μl of Solution A containing:

100 pmoles d(GGTTGGCCAATCTACTC-
CCAGG) primer
100 pmoles d(TAACCTTGATAC-
CAACCTGCCC) primer
1000 pmoles each of dATP, dCTP, dGTP and TTP

The two primers were prepared by the technique described in Example 2. The solution was heated to 100° C. for four minutes and allowed to cool in ambient air for two minutes, after which was added 1 μl containing four units Klenow fragment of *E. coli* DNA polymerase. The reaction was allowed to proceed for 10 minutes after which the cycle of solution A addition, heating, cooling, adding polymerase, and reacting was repeated three times. To a 5.0 μl aliquot of the reactions was added 5 picomoles of each oligonucleotide primer described above. The solution was heated to 100° C. for four minutes and allowed to come to ambient temperature, after which 3 picomoles each of the alpha-^{32}P-labeled deoxyribonucleoside triphosphates and 4 units Klenow fragment were added. The reaction, in a final volume of 10 μl and at the salt concentrations given

above, was allowed to proceed for 10 minutes. The polymerase activity was terminated by heating for 20 minutes at 60° C. Two μl aliquots were digested with NcoI, MstII, or HinfI and loaded onto a 12% polyacrylamide gel 0.089M in Tris-borate at pH 8.3 and 2.5 mM in EDTA. The gel was electrophoresed at 25 volts/cm for four hours and autoradiography was performed. FIG. 5 illustrates the autoradiograph of the electrophoresis, where Lane 1 is the molecular weight standard, Lane 2 is without digestion with enzyme (240 bp intact), Lane 3 is digestion with NcoI (131 and 109 bp), Lane 4 is digestion with MstII (149 and 91 bp), and Lane 5 is digestion with HinfI (144 and 96 bp). The autoradiograph is consistent with the amplification of the 240 bp sequence.

EXAMPLE 5

This example illustrates use of the process herein to detect sickle cell anemia by sequential digestion.

Synthesis and Phosphorylation of Oligodeoxyribonucleotides

A labeled DNA probe, RS06, of the sequence:

5′ *CTGACTCCTGAGGAGAAGTCTGCCGT-TACTGCCCTGTGGG 3′

where * indicates the label, and an unlabeled blocking oligomer, RS10, of the sequence:

3′ GACAGAGGTCACCTCTTCAGACG-GCAATGACGGGACACCC 5′

which has three base pair mismatches with RS06 were synthesized according to the procedures provided in Example 2(I). The probe RS06 was labeled by contacting five pmole thereof with 4 units of T4 polynucleotide kinase (New England Biolabs) and 50 pmole γ-^{32}P-ATP (New England Nuclear, about 7200 Ci/mmole) in a 40 μl reaction volume containing 70 mM Tris buffer (pH 7.6), 10 mM $MgCl_2$, 1.5 mM spermine, and 2.5 mM dithiothreitol for 90 minutes at 37° C. The total volume was then adjusted to 100 μl with 25 mM EDTA and purified according to the procedure of Maniatis et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory, 1982), pp. 464–465 over a 1 ml Bio Gel P-4 spin dialysis column from Bio-Rad equilibrated with Tris-EDTA (TE) buffer (10 mM Tris buffer, 0.1 mM EDTA, pH 8.0). The labeled probe was further purified by electrophoresis on a 18% polyacrylamide gel (19:1 acrylamide:BIS, Bio-Rad) in Tris-boric acid-EDTA (TBE) buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) for 500 vhr. After localization by autoradiography, the portion of the gel containing the labeled probe was excised, crushed and eluted into 0.2 ml TE buffer overnight at 4° C. TCA precipitation of the reaction product indicated that the specific activity was 4.9 Ci/mmole and the final concentration was 20 pmole/ml.

The labeled RS10 blocking oligomer was used at a concentration of 200 pmole/ml.

Isolation of Human Genomic DNA from Cell Lines

High molecular weight genomic DNA was isolated from the lymphoid cell lines Molt4, SC-1 and GM2064 using essentially the method of Stetler et al., *Proc. Natl. Acad. Sci.* USA (1982), 79, 5966–5970 (for Molt4) and Maniatis et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory, 1982), pp. 280–281.

Molt4 (Human Mutant Cell Repository, GM2219C) is a T cell line homozygous for normal β-globin, and SC-1, deposited with ATCC on Mar. 19, 1985, is an EBV-transformed B cell line homozygous for the sickle cell allele. GM2064 (Human Mutant Cell Repository, GM2064) was originally isolated from an individual homozygous for hereditary persistence of fetal hemoglobin (HPFH) and contains no beta- or delta-globin gene sequences. All cell lines were maintained in RPMI-1640 with 10% fetal calf serum.

Isolation of Human Genomic DNA from Clinical Blood Samples

A clinical blood sample designated CH12 from a known sickle cell carrier (AS) was obtained from Dr. Bertram Lubin of Children's Hospital in Oakland, Calif. Genomic DNA was prepared from the buffy coat fraction, which is composed primarily of peripheral blood lymphocytes, using a modification of the procedure described by Nunberg et al., *Proc. Nat. Acad. Sci. USA*, 75, 5553–5556 (1978).

The cells were resuspended in 5 ml Tris-EDTA-NaCl (TEN) buffer (10 mM Tris buffer pH 8, 1 mM EDTA, 10 mM NaCl) and adjusted to 0.2 mg/ml proteinase K, 0.5% SDS, and incubated overnight at 37° C. Sodium perchlorate was then added to 0.7M and the lysate gently shaken for 1–2 hours at room temperature. The lysate was extracted with 30 ml phenol/chloroform (1:1), then with 30 ml chloroform, and followed by ethanol precipitation of the nucleic acids. The pellet was resuspended in 2 ml of TE buffer and RNase A added to 0.005 mg/ml. After digestion for one hour at 37° C., the DNA was extracted once each with equal volumes of phenol, phenol/chloroform, and chloroform, and ethanol precipitated. The DNA was resuspended in 0.5 nm.

Polymerase Chain Reaction to Amplify Selectively β-Globin Sequences

Two micrograms of genomic DNA was amplified in an initial 100 μl reaction volume containing 10 mM Tris buffer (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 150 pmole of Primer A of the sequence d(CACAGGGCAC-TAACG), and 150 pmole of Primer B of the sequence d(CTTTGCTTCTGACACA) and overlayed with about 100 μl mineral oil to prevent evaporation.

Each DNA sample underwent 15 cycles of amplification where one cycle is composed of three steps:

(1) Denature in a heat block set at 95° C. for two minutes.

(2) Transfer immediately to a heat block set at 30° C. for two minutes to allow primers and genomic DNA to anneal.

(3) Add 2 μl of a solution containing 5 units of the Klenow frgment of *E. coli* DNA polymerase I (New England Biolabs), 1 nmole each of dATP, dCTP, dGTP and TTP, in a buffer composed of 10 mM Tris (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, and 4 mM dithiothreitol. This extension reaction was allowed to proceed for 10 minutes at 30° C.

After the final cycle, the reaction was terminated by heating at 95° C. for two minutes. The mineral oil was extracted with 0.2 ml of chloroform and discarded. The final reaction volume was 130 μl.

Hybridization/Digestion of Amplified Genomic DNA with Probes and DdeI/HinfI

Forty-five microliters of the amplified genomic DNA was ethanol precipitated and resuspended in an equal volume of TE buffer. Ten microliters (containing the

pre-amplification equivalent of 154 ng of genomic DNA) was dispensed into a 1.5 ml Microfuge tube and 20 μl of TE buffer to a final volume of 30 μl. The sample was overlayed with mineral oil and denatured at 95° C. for 10 minutes. Ten microliters of 0.6M NaCl containing 0.02 pmole of labeled RS06 probe was added to the tube, mixed gently, and immediately transferred to a 56° C. heat block for one hour. Four microliters of unlabeled RS10 blocking oligomer (0.8 pmole) was added and the hybridization continued for an additional 10 minutes at the same temperature. Five microliters of 60 mM $MgCl_2$/0.1% BSA and 1 μl of DdeI (10 units, New England Biolabs) were added and the reannealed DNA was digested for 30 minutes at 56° C. One microliter of HinfI (10 units, New England Biolabs) was then added and incubated for another 30 minutes. The reaction was stopped by the addition of 4 μl 75 mM EDTA and 6 μl tracking dye to a final volume of 61 μl.

The mineral oil was extracted with 0.2 ml chloroform, and 18 μl of the reaction mixture (45 ng genomic DNA) was loaded onto a 30% polyacrylamide mini-gel (19:1, Bio-Rad) in a Hoeffer SE200 apparatus. The gel was electrophoresed at approximately 300 volts for one hour until the bromophenol blue dye front migrated to 3.0 cm off-origin. The top 1.5 cm of the gel was removed and the remaining gel was exposed for four days with one intensification screen at −70° C.

Discussion of Photograph (FIG. 9)

Each lane contains 45 ng of amplified genomic DNA. Lane A contains Molt4 DNA; Lane B, CH12; Lane C, SC-1; and Lane D, GM2064. Molt4 represents the genotype of a normal individual with two copies of the β^A gene per cell (AA), CH12 is a clinical sample from a sickle cell carrier with one β^A and one β^S gene per cell (AS), and SC-1 represents the genotype of a sickle cell individual with two copies of the β^S gene per cell (SS), GM2064, which contains no beta- or delta-globin sequences, is present as a negative control.

As seen in the photograph, the DdeI-cleaved, β^A-specific octamer is present only in those DNA's containing the β^A gene (Lanes A and B), and the HinfI-cleaved, β^S-specific trimer is present only in those DNA's containing the β^S gene (Lanes B and C). The presence of both trimer and octamer (Lane B) is diagnostic for a sickle cell carrier and is distinguishable from a normal individual (Lane A) with only octamer and a sickle cell afflicted individual (Lane C) with only trimer.

As a comparison, repeating the experiment described above using non-amplified genomic, DNA revealed that the amplification increased the sensitivity of detection by at least 1000 fold.

EXAMPLE 6

This example illustrates direct detection of a totally unpurified single copy gene in which human DNA on gels without the need for a labeled probe.

Using the technique described in Example 3, a 110-bp fragment from a sequence in the first exon of the beta-globin gene was amplified from 10 micrograms of whole human DNA after 20 cycles. This 110-bp fragment produced after 20 cycles was easily visualized on gels stained with ethidium bromide.

The sequence was not amplified when it was first cut with the restriction enzyme DdeI unless, as in the beta-globin S allele, the sequence does not contain the restriction site recognized by the enzyme.

EXAMPLE 7

A. A total of 100 fmoles pBR328 containing a 1.9 kb insert from the human beta-globin A allele, 50 nmoles each alpha-32P-dNTP at 500 Ci/mole, and 1 nmole of each of the primers used in Example 3 were dissolved in a solution containing 100 μl 30 mM Tris-acetate at pH 7.9, 60 mM sodium acetate, 100 mM dithiothreitol, and 10 mM magnesium acetate. This solution was brought to 100° C. for two minutes and cooled to 25° C. for one minute. A total of 1 μl containing 4.5 units Klenow fregment of *E. coli* DNA polymerase I and 0.09 units inorganic pyrophosphatase was added to prevent the possible build-up of pyrophosphate in the reaction mixture, and the reaction was allowed to proceed for two minutes at 25° C., after which the cycle of heating, cooling, adding enzyme, and reacting was repeated nine times. Ten-μl aliquots were removed and added to 1 μl 600 mM EDTA after each synthesis cycle. Each was analyzed on a 14% polyacrylamide gel in 90 mM Tris-borate and 2.5 mM EDTA at pH 8.3 and 24 volts/cm for 2.5 hours. The completed gel was soaked for 20 minutes in the same buffer with the addition of 0.5 μg/ml ethidium bromide, washed with the original buffer, and photographed in UV light using a red filter.

The 110-bp fragment produced was excised from the gel under ultraviolet light and the incorporated ^{32}P counted by Cerenkov radiation. An attempt to fit the data to an equation of the form: pmoles/10 μl=0.01 $[(1+y)^N-yN-1]$, where N represents the number of cycles and y the fractional yield per cycle, was optimal with y=0.619. This indicates that a significant amplification is occurring.

B. The above experiment was repeated except that 100 nmoles of each dNTP was added to a 100 μl reaction, no radiolabel was employed, and aliquots were not removed at each cycle. After 10 cycles the reaction was terminated by boiling for two minutes and rehybridization was performed at 57° C. for one hour. The sequence of the 110-bp product was confirmed by subjecting 8 μl aliquots to restriction analysis by addition of 1 μl bovine serum albumin (25 mg/ml) and 1 μl of the appropriate restriction enzyme (HinfI, MnlI, MstII, NcoI) and by reaction at 37° C. for 15 hours. PAGE was performed as described above.

EXAMPLE 8

This example illustrates the use of different primers to amplify various fragments of pBR328 and 322.

A. The experiment described in Example 7A was repeated except using the following primers: d(TTTGCTTCTGACACAACTGTGTTCACTAGC) and d(GCCTCACCACCAACTTCATCCACGTTCACC) to produce a 130-bp fragment of pBR328.

B. The experiment described in Example 7A was repeated except using the following primers: d(GGTTGGCCAATCTACTCCCAGG) and d(TGGTCTCCTTAAACCTGTCTTG) to produce a 262-bp fragment of pBR328. The reaction time was 20 minutes per cycle.

The experiment described in Example 8B was repeated except that 100 fmoles of an MstII digest of pBR328 containing a 1.9 kb insert from the human beta-globin S allele was used as initial template. This plasmid was cleaved several times by MstII but not inside the sequence to be amplified. In addition, the primers employed were as follows:

d(GGTTGGCCAATCTACTCCCAGG) and
d(TAACCTTGATACCAACCTGCCC)
to produce a 240-bp fragment.

D. The experiment described in Example 7B was repeated except that 100 fmoles of an NruI digest of pBR322 was used as template, 200 nmoles of each dNTP were used in the 100 μl reaction, and the primers were:
d(TAGGCGTATCACGAGGCCCT) and
d(CTTCCCCATCGGTGATGTCG)
to produce a 500-bp fragment from pBR322. Reaction times were 20 minutes per cycle at 37° C. Final rehybridization was 15 hours at 57° C. Electrophoresis was on a 4% agarose gel.

EXAMPLE 9

This example illustrates the invention process wherein an in vitro mutation is introduced into the amplified segment.

A. A total of 100 fmoles of pBR322 linearized with NruI, 1 nmole each of the primers:
d(CGCATTAAAGCTTATCGATG) and
d(TAGGCGTATCACGAGGCCCT)
designed to produce a 75-bp fragment, 100 nmole each dNTP, in 100 μl 40 mM Tris at pH 8, 20 mM in $MgCl_2$, 5 mM in dithiothreitol, and 5 mg/ml bovine serum albumin were combined. The mixture was brought to 100° C. for one minute, cooled for 0.5 minutes in a water bath at 23° C., whereupon 4.5 units Klenow fragment and 0.09 units inorganic pyrophosphatase were added, and a reaction was allowed to proceed for three minutes. The cycle of heating, cooling, adding enzymes, and reacting was repeated nine times. The tenth reaction cycle was terminated by freezing and an 8-μl aliquot of the reaction mixture was applied to a 4% agarose gel visualized with ethidium bromide.

B. The experiment described in Example 9A was repeated except that the oligonucleotide primers employed were:
d(CGCATTAAAGCTTATCGATG) and
d(AATTAATACGACTCACTATAGG-
GAGATAGGCGTATCACGAGGCCCT).
Thse primers are designed to produce a 101-bp fragment, 26 nucleotides of which (in the second listed primer) are not present in pBR322. These nucleotides represent the sequence of the T7 promoter, which was appended to the 75-bp sequence from pBR322 by using the primer with 20 complementary bases and a 26-base 5′ extension. The procedure required less than two hours and produced two picomoles of the relatively pure 101-bp fragment from 100 fmoles of pBR322.

The T7 promoter can be used to initiate RNA transcription. T7 polymerase may be added to the 101-bp fragment to produce single-stranded RNA.

C. The experiment described in Example 8D was repeated except that the olignonucleotide primers employed were as follows:
d(TAGGCGTATCACGAGGCCCT) and
d(CCAGCAAGACGTAGCCCAGC)
to produce a 1000-bp fragment from pBR322.

D. The experiment described in Example 9C was repeated except that the oligonucleotide primers employed were as follows:
d(TAGGCGTATCACGAGGCCCT) and
d(AATTAATACGACTCACTATAGG-
GAGATAGGCGTATCACGAGGCCCT)
so as to produce a 1026-bp fragment, 26 nucleotides of which (in the second listed primer) are not present in pBR322 and represent the T7 promoter described above. The promoter has been inserted adjacent to a 1000-bp fragment from pBR322.

The results indicate that a primer which is not a perfect match to the template sequence but which is nonetheless able to hybridize sufficiently to be enzymatically extended produces a long product whwich contains the sequence of the primer rather than the corresponding sequence of the original template. The long product serves as a template for the second primer to introduce an in vitro mutation. In further cycles this mutation is amplified with an undiminished efficiency, because no further mispaired primings are required. In this case, a primer which carries a non-complementary extension on its 5′ end was used to insert a new sequence in the product adjacent to the template sequence being copied.

EXAMPLE 10

This example illustrates employing nested sets of primers to decrease the background in the amplification of single copy genes.

Whole human DNA homozygous for the wild-type β-globin allele was subjected to twenty cycles of amplification as follows: A total of 10 μg DNA, 200 picomoles each of the primers:
d(ACACAACTGTGTTCACTAGC) and
d(CAACTTCATCCACGTTCACC)
and 100 nanomoles each dNTP in 100 μl of 30 mM Tris-acetate pH 7.9, 60 mm sodium acetate, 10 mM dithiothreitol, and 10 mM magnesium acetate were heated to 100° C. for one minute, cooled to 25° C. for one minute, and treated with 2 units Klenow fragment for two minutes. The cycle of heating, cooling and adding Klenow as repeated 19 times. A ten-μl aliquot was removed from the reaction mixture and subjected to a further ten cycles of amplification using each of the primers:
d(CAGACACCATGGTGCACCTGACTCCTG)
and
d(CCCCACAGGGCAGTAACG-
GCAGACTTCTCC),
which amplify a 58-bp fragment contained within the 110-bp fragment produced above. This final ten cycles of amplification was accomplished by diluting the 10-μl aliquot into 90 μl of the fresh Tris-acetate buffer described above containing 100 nanomoles each dNTP and 200 pmoles of each primer. Reaction conditions were as above. After ten cycles a 10-μl aliquot (corresponding to 100 nanograms of the original DNA) was applied to a 6% NuSieve (FMC Corp.) agarose gel and visualized using ethidium bromide.

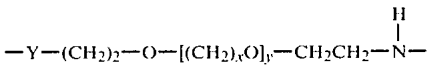
FIG. **10** illustrates this gel illuminated with UV light and photographed through a red filter as is known in the art. Lane 1 is molecular weight markers. Lane 2 is an aliquot of the reaction described above. Lane 3 is an aliquot of a reaction identical to that described above, except that the original wild-type DNA was cleaved with DdeI prior to amplification. Lane 4 is an aliquot of a reaction identical to that described above, except that human DNA homozygous for the sickle betaglobin allele was treated with DdeI prior to amplification (the sickle allele does not contain a DdeI site in the fragment being amplified here). Lane 5 is an aliquot of a reaction identical to that described above, except that salmon sperm DNA was substituted for human DNA. Lane 6 is an aliquot of a reaction identical to that described above, except that the aliquot was treated with DdeI

after amplification (DdeI should convert the 58-bp wild-type product into 27- and 31-bp fragments). Lane 7 is an aliquot of the Lane 4 material treated with DdeI after amplification (the 58-bp sickle product contains no DdeI site).

Detection of a 58-bp fragment representative of a single-copy gene from one microgram of human DNA using only ethidium bromide staining of an agarose gel requires an amplification of about 500,000-fold. This was accomplished by using the two nested sets of oligonucleotide primers herein. The first set amplifies the 110-bp fragment and the inner nested set amplifies a sub-fragment of this product up to the level of convenient detection shown in FIG. **10**. This procedure of using primers amplifying a smaller sequence contained within the sequence being amplified in the previous amplification process and contained in the extension products of the other primers allows one to distinguish the wild-type from the sickle allele at the betaglobin locus without resorting to either radioisotropic or non-radioisotopic probe hybridization methodology such as that of Conner et al., *Proc. Natl. Acad. Sci.* USA, 80:278 (1983) and Leary et al., *Proc. Natl. Acad. Sci.* USA, 80:4045 (1983).

EXAMPLE 11

The present process is expected to be useful in detecting, in a patient DNA sample, a specific sequence associated with an infectious disease such as, e.g., Chlamydia using a biotinylated hybridization probe spanning the desired amplified sequence and using the process described in U.S. Pat. No. 4,358,535, supra. The biotinylated hybridization probe may be prepared by intercalation and irradiation of a partially double-stranded DNA with a 4'-methylene substituted 4,5'-8-trimethylpsoralen attached to biotin via a spacer arm of the formula:



where Y is O, NH or N—CHO, x is a number from 1 to 4, and y is a number from 2 to 4, as described in U.S. Pat. Nos. 4,582,789 issued Apr. 15, 1986 and 4,617,261 issued Oct. 14, 1986, the disclosures of which are incorporated herein by reference. Detection of the biotinyl groups on the probe may be accomplished using a streptavidin-acid phosphatase complex commercially obtainable from Enzo Biochemical using the detection procedures suggested by the manufacturer in its brochure. The hybridized probe is seen as a spot of precipitated stain due to the binding of the detection complex, and the subsequent reaction catalyzed by acid phosphatase, which produces a precipitable dye.

EXAMPLE 12

In this example, the process of Example 7 was basically used to amplify a 119 base pair fragment on the human β-hemoglobin gene using the primers:

5'-CTTCTGcagCAACTGTGTTCACTAGC-3' (GH18)

5'-CACaAgCTTCATCCACGTTCACC-3' (GH19)

where lower case letters denote mismatches from wild-type sequence to create restriction enzyme sites. The full scheme is shown in Table I. Table I illustrates a diagram of the primers GH18 and GH19 which are used for cloning and sequencing a 119-base pair fragment of the human β-globin gene and which are designed to contain internal restriction sites. The start codon ATG is underlined. GH18 is a 26-base oligonucleotide complementary to the negative strand and contains an internal PstI site. GH19 is a 23-base oligonucleotide complementary to the plus strand and contains an internal HindIII recognition sequence. Arrows indicate the direction of extension by DNA polymerase I. The boxed sequences indicate the restriction enzyme recognition sequences of each primer. These primers were selected by first screening the regions of the gene for homology to the PstI and HindIII restriction sites of bacteriophage M13. The primers were then prepared as described in previous examples.

TABLE I

DdeI

GH19

←CCACTTGCACCTAC[TTCgAa]CAC

CTTCTGACACAACTGTGTTCACTAGCAACCTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCCGTTACTGCCCTGTGGGGCAAGGTGAACGTGGATGAAGTTGGTG(+)

GAAGACTGTGTTGACACAAGTGATCGTTGGAGTTTGTCTGTGGTACCACGTGGACTGAGGACTCCTCTTCAGACGGCAATGACGGGACACCCCGTTCCACTTGCACCTACTTCAACCAC(−)

CTTCTG[cagCAA]CTGTGTTCACTAGC→

GH18

Pst I

5′ CTTCTG[cagCAA]CTGTGTTCACTAGC 3′ GH18 left linker primer

5′ CAC[aAgCTT]CATCCACGTTCACC 3′ GH19 roght linker primer

Hind III

Amplification and Cloning

After twenty cycles of amplification of 1 microgram of human genomic DNA isolated from the cell line Molt 4 as described in Example 2, 1/14th of the reaction product was hybridized to the labeled β-globin specific oligonucleotide probe, RS06, of the sequence 5'-CTGACTCCTGAGGAGAAGTCTGCCGT-TACTGCCCTGTGGG-3' using the methods described above for oligomer restriction. Following solution hybridization, the reaction mixture was treated with DdeI under restriction digestion conditions as described above, to produce an 8-base pair oligonucleotide. The amount of this 8-base pair product is proportional to the amount of amplified product produced. The digestion products were resolved on a 30% polyacrylamide gel and visualized by autoradiography.

Analysis of the autoradiogram revealed that the amplification was comparable in efficiency to that of amplification with primers PC03 (5'-ACACAACTGTGTTCACTAGC-3') and PC04 (5'-CCACTTGCACCTACTTCAAC-3'), which are complementary to the negative and positive strands, respectively, of the wild-type β-globin.

The amplified product was ethanol precipitated to desalt and concentrate the sample, redissolved in a restriction buffer of 10 mM Tris pH 8, 10 mM $MgCl_2$, 1 mM DTT, 100 mM $NaCl_2$, and simultaneously digested with PstI and HindIII. After digestion the sample was desalted with a Centricon 10 concentrator and ligated overnight at 12° C. with 0.3 micrograms of the PstI/-HindIII digested vector M13mp10w, which is publicly available from Boehringer-Mannheim.

The entire ligation mixture was transformed into *E. coli* strain JM103, which is publicly available from BRL in Bethesda, MD. The procedure followed by preparing the transformed strain is described in Messing, J. (1981) *Third Cleveland Symposium on Macromolecules:Recombinant DNA,* ed. A. Walton, Elsevier, Amsterdam, 143–153.

The transformation mixture was plated onto x-gal media for screening via plaque hybridization with nylon filters. The filters were probed with a β-globin-specific oligonucleotide probe RS24 of the sequence 5'-CCCACAGGGCAGTAACGGCAGACTTCTCCT-CAGGAGTCAG-3' to determine the number of β-globin inserts. The filters were then reprobed with the primer PC04 to determine the total number of inserts.

Plating and Screening

Table II summarizes the plating and plaque hybridization data. The filters were probed with the primer PC04 to determine the percentage of inserts resulting from amplification and cloning; 1206 clear plaques (90% of total number of clear plaques) hybridized to the primer. Fifteen plaques hybridized to the β-globin specific probe RS24. The percentage of β-globin positive plaques among the amplified primer-positive plaques is approximately 1%.

TABLE II

Plate No.	Blue Plaques	No Inserts*	Inserts**	β-Globin Inserts
1	28	25	246	1
2	29	18	222	2
3	11	26	180	0
4	24	20	192	5
5	22	27	185	5
6	39	21	181	3
TOTAL	158	132	1206	15

% of plaques containing amplified sequences which contain β-globin insert = 15/1206 × 100 = 1.24%
% of total plaques which contain β-globin insert = 15/1496 × 100 = ca.1%
% of total plaques which contain amplified sequences = 1206/1496 × 100 = 0.8%
*Clear plaques which do not hybridize to primer PC04
**Clear plaques which hybridize to primer PC04

Restriction Enzyme and Southern Blot Analysis

DNAs from phage DNA minipreparation of three β-globin positive and two β-globin negative (but PC04 primer positive) plaques were analyzed by restriction enzyme analysis. MstII digestion of DNA from M13 clones containing the amplified β-globin fragment should generate a characteristic 283 base-pair fragment. Following MstII digestion, the three β-globin positive clones all produced the predicted 283 base pair fragment, while the two clones which were positive only with the primer produced larger fragments.

The gel from this analysis was transferred to a MSI nylon filter and hybridized with a radiolabeled nick-translated β-globin probe prepared by standard nick translation methods as described by Rigby et al., *J. Mol. Biol.* (1977), 113:237–51. The only bands which hybridized to the β-globin probe were the three β-globin positive clones. The two other clones had inserts which did not hybridize to the β-globin probe.

Sequence Analysis

Ten β-globin positive clones which were shown by restriction enzyme analysis to contain the β-globin insert were sequenced using the M13-dideoxy sequencing method. Of the ten clones, nine were identical to the β-globin wild-type sequence. The other clone was identical to the ∂-globin gene which had been shown to be amplified to only a small degree by the β-globin primers.

In conclusion, the modified linker primers were nearly as efficient as the unmodified primers in amplifying the β-globin sequence. The primers were able to facilitate insertion of amplified DNA into cloning vectors. Due to the amplification of other segments of the genome, only 1% of the clones contained hemoglobin sequences.

Nine of the ten clones were found to be identical to the published β-globin sequence, showing that the technique amplifies genomic DNA with high fidelity. One clone was found to be identical with the published ∂-globin sequence, confirming that the primers are specific for the β-globin gene despite their having significant sequence homology with δ-globin.

When cloning was carried out with a 267 base pair fragment of the β-globin gene, cloning was effective only when dimethylsulfoxide was present (10% by volume at 37° C.) in the amplification procedure.

Restriction site-modified primers were also used to amplify and clone and partially sequence the human N-ras oncogene and to clone 240-base pair segments of the HLA DQ-α and DQ-β genes. All of these amplifications were carried out in the presence of 10% by volume dimethylsulfoxide at 37° C. The primers for amplifying HLA DQ-α and DQ-β genes were much more specific for their intended targets than were the β-globin and DR-β primers, which, rather than giving a

discrete band on an ethidium bromide stained agarose gel, produced only a smear. In addition, the HLA DQ-α primers produced up to 20% of clones, with amplified inserts which contained the desired HLA target fragment, whereas 1% of the β-globin clones contained the target sequence. The HLA DQ-α and DQ-β gene cloning was only effective when the DMSO was present and the temperature was elevated.

EXAMPLE 13

This example illustrates the use of the process herein to prepare the TNF gene of 494 base pairs starting from two oligonucleotides of 74 base pairs each.

PRIMERS

The primers employed were prepared by the method described in Example 2 and are identified below, each being 74 mers.

(TN10) 5'-CCTCGTCTACTCCCAGGTCCTCTT-CAAGGGCCAAGGCTGCCCCGAC-TATGTGCTCCTCACCCACACCGTCAGCC-3'

(TN11) 5'-GGCAGGGGCTCTTGACG-GCAGAGAGGAGGTTCACCTTCTCCTG-GTAGGAGATGGCGAAGCGGCT-GACGGTGTGG-3'

(LL09) 5'-CCTGGCCAATGGCATGGATCT-GAAAGATAACCAGCTGGTGGTGCCAG-CAGATGGCCTGTACCTCGTCTACTCCC-3'

(LL12) 5'-CTCCCTGATAGATGGGCTCATAC-CAGGGCTTGAGCT-CAGCCCCCTCTGGGGTGTCCTTCGG-GCAGGGGCTCTTG-3'

(TN08) 5'-TGTAGCAAACCATCAAGTTGAG-GAGCAGCTCGAGTGGCTGAGC-CAGCGGGCCAATGCCCTCCTGG-CCAATGGCA-3'

(TN13) 5'-GATACTTGGGCAGATTGACCT-CAGCGCTGAGTTGGTCACCCTTCT-CCAGCTGGAAGACCCCTCCCT-GATAGATG-3'

(LL07) 5'-CCTTAAGCTTATGCTCAGAT-CATCTTCTCAAAACTCGAGT-GACAAGCCTGTAGCCCATGTTGTAG-CAAACCATC-3'

(TN14) 5'-GCTCGGATCCTTACAGGGCAAT-GACTCCAAAGTAGACCTGC-CCAGACTCGGCAAAGT-CGAGATACTTGGGCAGA-3'

OVERALL PROCEDURE

I. Ten cycles of the protocol indicated below were carried out using primers TN10 and TN11, which interact as shown in the diagram below, step (a).

II. A total of 2 μl of the reaction mixture from Part I above was added to the primers LL09 and LL12. The protocol described below was carried out for 15 cycles, so that the primers would interact with the product of Part I as shown in the diagram below, step (b).

III. A total of 2 μl of the reaction mixture from Part II above was added to the primers TN08 and TN13. The protocol described below was carried out for 15 cycles, so that the primers would interact with the product of Part II as shown in the diagram below, step (c).

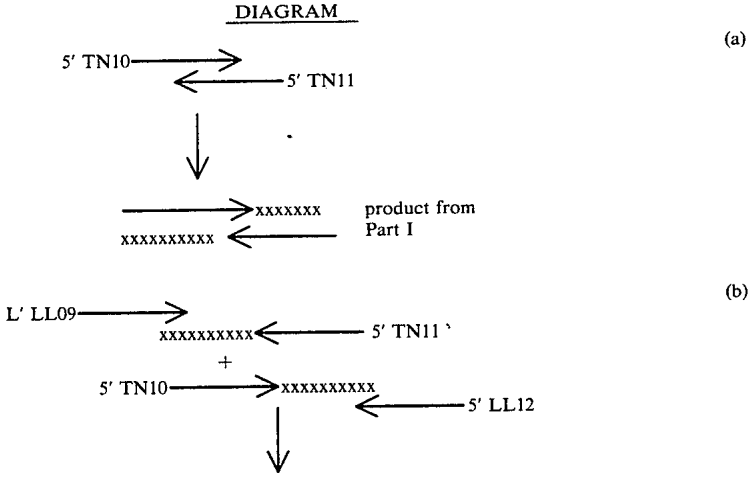
IV. A total of 2 μl of the reaction mixture from Part III above was added to the primers LL07 and LL14. The protocol described below was carried out for 15 cycles, so that the primers would interact with the product of Part III as shown in the diagram below, step (d).

PROTOCOL

Each reaction contained 100 μl of:
- 2 mM of each of dATP, dCTP, dGTP and TTP
- 3 μM of each of the primers used at that step
- 1×polymerase buffer, (30 mM Tris-acetate, 60 mM Na-acetate, 10 mM Mg-acetate, 2.5 mM dithiothreitol)

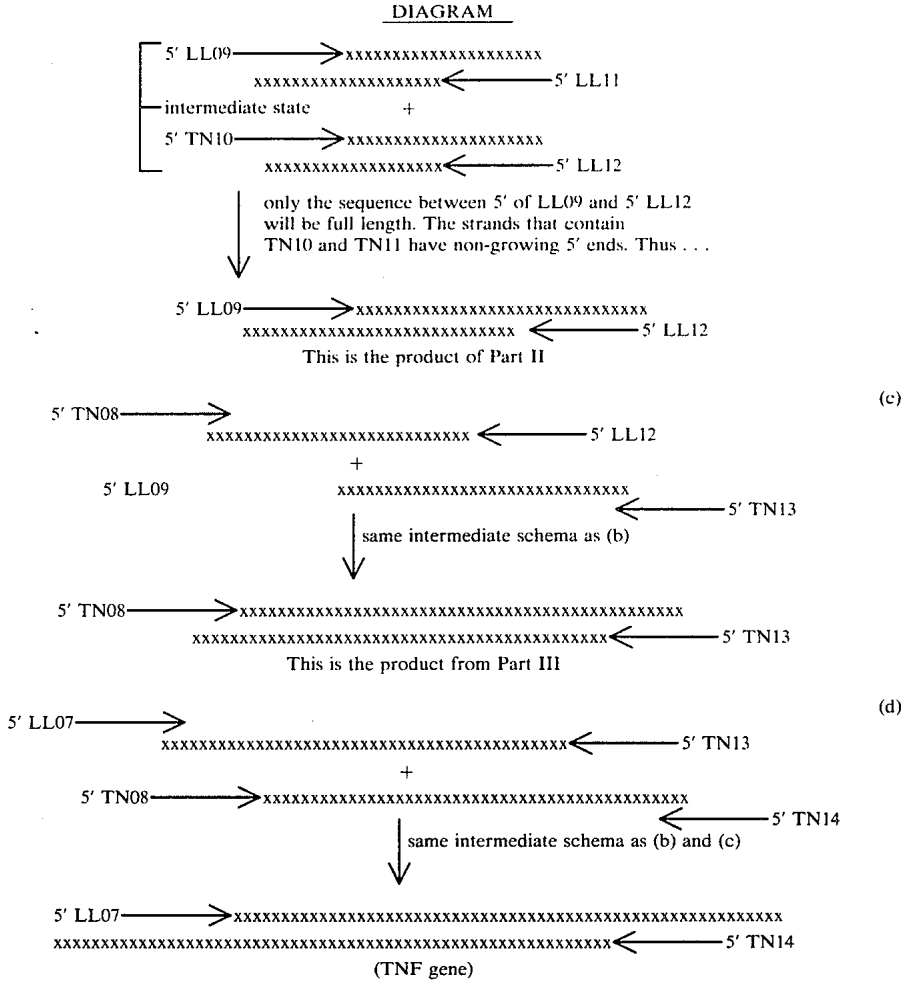
Each cycle constituted:
- (1) 1 min. in boiling water
- (2) 1 min. cooling at room temperature
- (3) add 1 μl (5 units) of the Klenow fragment of DNA polymerase
- (4) allow the polymerization reaction to proceed for 2 min. For the next cycle start again at step 1.

DIAGRAM



-continued

DIAGRAM



Deposit of Materials

The cell line SC-1 (CTCC #0082) was deposited on Mar. 19, 1985 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852 USA, with ATCC Accession No. CRL#8756. The deposit of SC-1 was made pursuant to a contract between the ATCC and the assignee of this patent application, Cetus Corporation. The contract with ATCC provides for permanent availability of the progeny of this cell line to the public on the issuance of the U.S. patent describing and identifying the deposit or the publications or upon the laying open to the public of any U.S. or foreign patent application, whichever comes first, and for availability of the progency of this cell line to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 CFR §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the cell line on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable culture of the same cell line.

In summary, the present invention is seen to provide a process for detecting sequences in nucleic acids by first amplifying one or more specific nucleic acid sequences using a chain reaction in which primer extension products are produced which can subsequently act as templates for further primer extension reactions. The process is especially useful in detecting nucleic acid sequences which are initially present in only very small amounts. Also, the amplification process can be used for molecular cloning.

Other modifications of the above described embodiments of the invention which are obvious to those of skill in the area of molecular biology and related disciplines are intended to be within the scope of the following claims.

What is claimed is:

1. A process for detecting the presence or absence of at least one specific nuclei acid sequence in a sample containing a nucleic acid or mixture of nucleic acids, or distinguishing between two different sequences in said sample, wherein the sample is suspected of containing said sequence or sequences, which process comprises:

(a) treating the sample with one oligonucleotide primer for each strand of each different specific sequence, under hybridizing conditions such that for each strand of each different sequence to which an oligonucleotide primer is hybridized an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primer or primers are selected so as to be sufficiently complementary to each strand of each specific sequence to hybridize therewith such

that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer;

(b) treating the sample under denaturing conditions to separate the primer extension products from their templates if the sequence or sequences to be detected are present;

(c) treating the sample with olignonucleotide primers such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, resulting in amplification of the specific nucleic acid sequence or sequences if present;

(d) adding to the product of step (c) a labeled oligonucleotide probe for each sequence being detected capable of hybridizing to said sequence or a mutation thereof; and

(e) determining whether said hybridization has occurred.

2. The process of claim **1**, wherein steps (b) and (c) are repeated at least once.

3. The process of claim **1**, wherein steps (a) and (c) are accomplished by treatment with four different nucleoside triphosphates and an agent for polymerization, which are added together with or separately from the primer(s).

4. The process of claim **1**, wherein said nucleic acid is double stranded and its strands are separated by denaturing before or during step (a).

5. The process of claim **1**, wherein said nucleic acid is single stranded.

6. The process of claim **4**, wherein said nucleic acid is DNA and said primers are oligodeoxyribonucleotides.

7. The process of claim **4**, wherein said nucleic acid is RNA and said primers are oligodeoxyribonucleotides.

8. The process of claim **5**, wherein said nucleic acid is DNA and said primers are oligodeoxyribonucleotides.

9. The process of claim **5**, wherein said nucleic acid is RNA and said primers are oligodeoxyribonucleotides.

10. The process of claim **1**, wherein each primer employed contains a restriction site on its 5′ end which is the same as or different from a restriction site on another primer, and after step (c) and before step (d) the product of step (c) is cleaved with a restriction enzyme specific for each of said restriction sites and the cleaved products are separated from the uncleaved products and used in step (d).

11. The process of claim **1**, wherein the specific nucleic acid sequence contains at least one specific deletion or mutation that causes a genetic disease.

12. The process of claim **11**, wherein the genetic disease is sickle cell anemia.

13. The process of claim **11**, wherein after step (c) and before step (d) the treated sample is cut with a restriction enzyme and electrophoresed and step (e) is accomplished by Southern blot analysis.

14. The process of claim **1**, wherein the specific nucleic acid sequence is contained in a pathogenic organism or is contained in an oncogene.

15. The process of claim **1**, wherein steps (a) and (c) are accomplished using an enzyme selected from the group consisting of *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, reverse transcriptase wherein the template is RNA or DNA and the extension product is DNA, and an enzyme that after being exposed to a temperature of about 65°–90° C. forms said extension products at the temperature of reaction during steps (a) and (c).

16. A process for detecting the presence or absence of a nucleic acid sequence containing a polymorphic restriction site specific for sickle cell anemia which sequence is suspected of being contained in a sample, which process comprises:

(a) treating the sample, together or separately, with an oligodeoxyribonucleotide primer for each strand, four different nucleoside triphosphates, and an agent for polymerization under hybridizing conditions, such that for each strand of the nucleic acid sequence an extension product of each primer is synthesized which is sufficiently complementary to each strand of the nucleic acid sequence being detected to hybridize therewith and contains the region of the β-globin gene known potentially to contain the mutation that causes sickle cell anemia, wherein said primers are selected such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer;

(b) treating the sample under denaturing conditions to separate the primer extension products from the templates on which they are synthesized if the sequence to be detected is present;

(c) treating the product of step (b) with oligodeoxyribonucleotide primers, four different nucleoside triphosphates, and an agent polymerization such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, resulting in amplification of the sequence to be detected if present;

(d) hybridizing said primer extension products of step (c) with a labeled oligodeoxyribonucleotide probe complementary to a normal β-globin gene;

(e) digesting the hybridized mixture from step (d) with a restriction enzyme for the restriction site specific for sickle cell anemia; and

(f) detecting whether the digest contains a restriction fragment correlated with the presence of sickle cell anemia.

17. The process of claim **16**, wherein in step (d) the probe spans DdeI and HinfI restriction sites, in step (e) the restriction enzyme is DdeI, and after step (e) and before step (f) the mixture is digested with restriction enzyme HinfI.

18. The process of claim **16**, wherein in steps (d)–(f) are present a positive control which contains a nucleic acid with the polymorphic restriction site specific for sickle cell anemia and a negative control which does not contain such nucleic acid.

19. A process for synthesizing a nucleic acid fragment from an existing nucleic acid fragment having fewer nucleotides than the fragment being synthesized and two oligonucleotide primers, wherein the nucleic acid being synthesized is comprised of a left segment, a core segment and a right segment, and wherein the core segment is sufficiently complementary to the nucleotide sequence of said existing nucleic acid fragment to hybridize therewith, and the right and left segments represent the nucleotide sequence present in the 5′ ends of the two primers, the 3′ ends of which are complementary to, or sufficiently complementary to hybridize with, the 3′ ends of the single strands produced by separating the strands of said existing nucleic acid fragment, which process comprises:

(a) treating the strands of said existing fragment with two oligonucleotide primers under conditions such

that an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primers are selected so as to be sufficiently complementary to the 3′ end of each strand of said existing fragment to hybridize therewith, such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein each primer contains, at its 5′ end, a sequence of nucleotides which are not complementary to said existing fragment and which correspond to the two ends of the nucleic acid fragment being synthesized;

(b) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

(c) treating the single-stranded molecules generared from step (b) with the primers of step (a) under conditions such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template so as to produce two intermediate double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ end of one of the oligonucleotide primers, and two full-length double-stranded nucleic acid molecules, into each of which has been incorporated the nucleotide sequence present in the 5′ ends of both of the oligonucleotide primers;

(d) repeating steps (b) and (c) for a sufficient number of times to produce the full-length double-stranded molecule in an effective amount;

(e) treating the strands of the product of step (d) with two primers so as to lengthen the product of step (d) on both ends; and

(f) repeating steps (a)–(d) using the product of step (d) as the core fragment and two oligonucleotide primers which are complementary to, or sufficiently complementary to hybridize with, the 3′ ends of the single strands produced by separating the strands of the product of step (d).

20. The process of claim **19**, wherein steps (b) and (c) are repeated at least five times.

21. The process of claim **20**, wherein the core segment used is the product of step (f).

22. The process of claim **19**, wherein the core fragment used is obtained by the steps comprising:

(a) reacting two oligonucleotides, each of which contain at their 3′ ends a nucleotide sequence which is complementary to the other oligonucleotide at its 3′ end, and which are non-complementary to each other at their 5′ ends, with an agent for polymerization and four nucleoside triphosphates under conditions such that an extension product of each oligonucleotide is synthesized which is complementary to each nucleic acid strand;

(b) separating the extension products from the templates on which they were synthesized to produce single-stranded molecules; and

(c) treating the single-stranded molecules generated from step (b) with the oligonucleotides of step (a) under conditions such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, resulting in amplification of the core fragment.

23. The process of claim **19**, wherein the product of step (d) is purified before step (e).

24. The process of claim **19**, wherein the product of step (d) is not purified before step (e).

25. The process of claim **19**, wherein steps (a) and (c) are accomplished by treatment with four different nucleoside triphosphates and an agent for polymerization, which are added together with or separately from the primers.

26. A process for cloning into bacteriophage M13 a polymorphic genetic sequence on the human HLA DQ, DR or DP Class Ii α and β genes, which process comprises:

(a) treating a genetic sequence of human HLA DQ, DR, or DP Class II α and β genes with one oligonucleotide primer for each strand of said sequence, under conditions such that for each strand an extension product of each primer is synthesized which is complementary to each nucleic acid strand, wherein said primers are selected so as to be sufficiently complementary to each strand to hybridize therewith such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer, and wherein each of said primers contains a restriction site on its 5′ end which is different from the restriction site on the other primer;

(b) separating the primer extension products from the templates on which they were synthesized to produce single-stranded molecules;

(c) treating the single-stranded molecules generated from step (b) with oligonucleotide primers such that a primer extension product is synthesized using each of the single strands produced in step (b) as a template, wherein steps (a) and (c) are carried out in the presence of an effective amount of dimethylsulfoxide to amplify sufficiently the amount of sequence produced and at a temperature of 35°–40° C.;

(d) adding to the product of step (c) a restriction enzyme for each of said restriction sites to obtain cleaved products in a restriction digest; and

(e) ligating the cleaved products into said bacteriophage M13 with a specific orientation.

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