Exhibit A

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

(10) **Patent No.: US 6,797,470 B2**
(45) **Date of Patent: Sep. 28, 2004**

(54) **DETECTION OF NUCLEIC ACID SEQUENCE DIFFERENCES USING COUPLED LIGASE DETECTION AND POLYMERASE CHAIN REACTIONS**

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(73) Assignees: **Cornell Research Foundation, Inc.**, Ithaca, NY (US); **Board of Supervisors of Louisiana State University and Agricultural and Mechanical College**, Baton Rouge, LA (US); **Regents of the University of Minnesota**, Minneapolis, MN (US)

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

(21) Appl. No.: **09/918,156**

(22) Filed: **Jul. 30, 2001**

(65) **Prior Publication Data**

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Related U.S. Application Data

(60) Continuation of application No. 09/440,523, filed on Nov. 15, 1999, now Pat. No. 6,268,148, which is a division of application No. 08/864,473, filed on May 28, 1997, now Pat. No. 6,027,889.

(60) Provisional application No. 60/018,532, filed on May 29, 1996.

(51) **Int. Cl.**[7] **C12Q 1/68**; C12P 19/34

(52) **U.S. Cl.** **435/6**; 435/91.1; 435/91.2

(58) **Field of Search** 435/6, 91.2, 91.1

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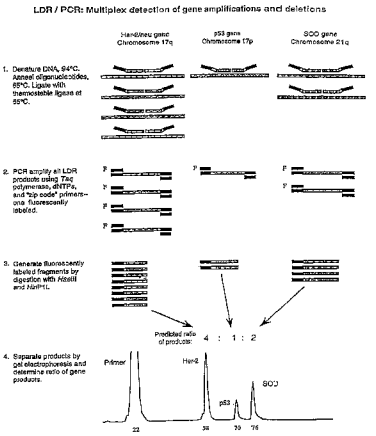
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(57) **ABSTRACT**

The present invention relates to the detection of nucleic acid sequence differences using coupled ligase detection reaction and polymerase chain reaction. One aspect of the present invention involves use of a ligase detection reaction coupled to a polymerase chain reaction. Another aspect of the present invention relates to the use of a primary polymerase chain reaction coupled to a secondary polymerase chain reaction coupled to a ligase detection reaction. A third aspect of the present invention involves a primary polymerase chain reaction coupled to a secondary polymerase chain reaction. Such coupling of the ligase detection reaction and the polymerase chain reaction permits multiplex detection of nucleic acid sequence differences.

26 Claims, 29 Drawing Sheets



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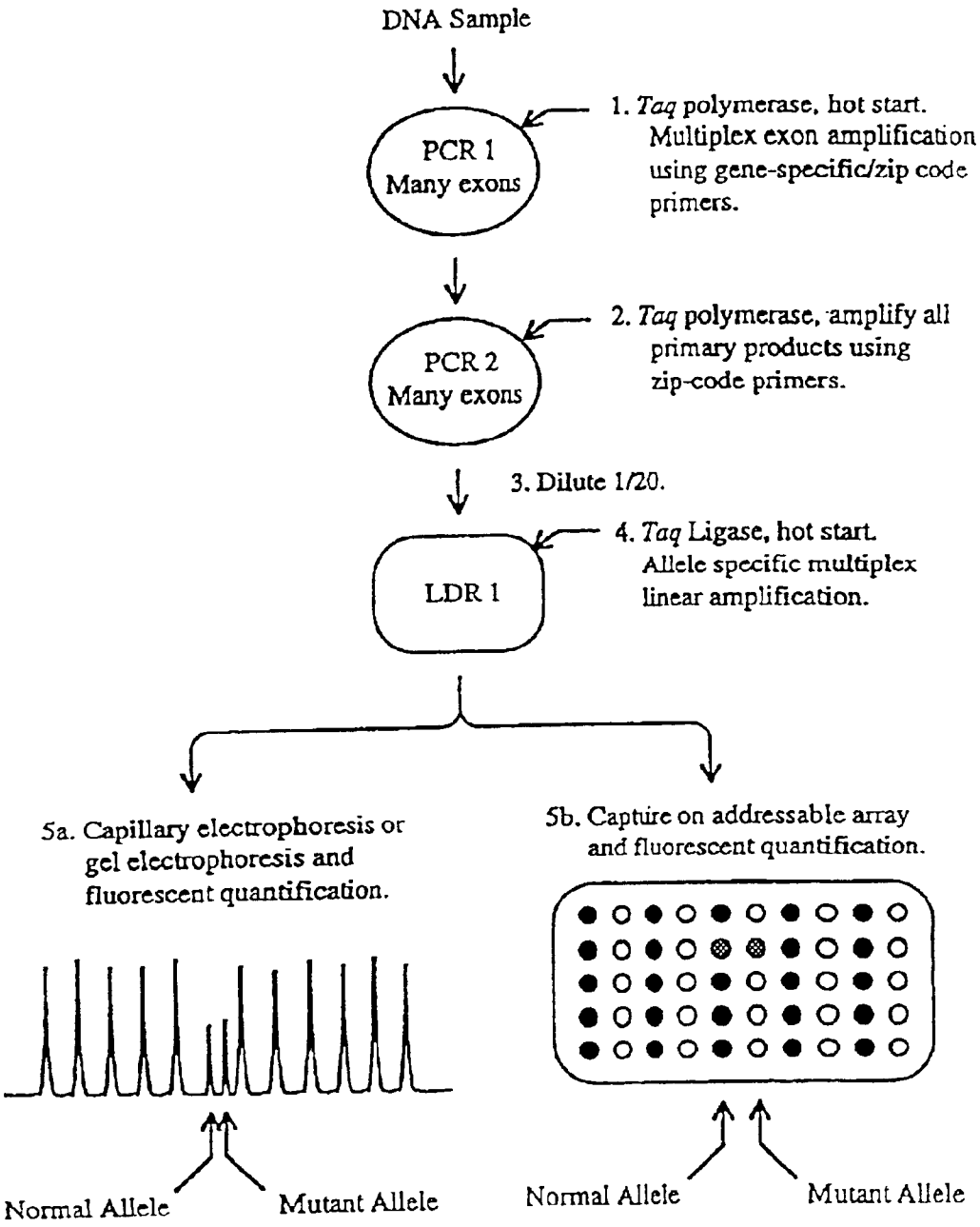

DNA Sample
PCR 1
Many exons
1. Taq polymerase, hot start. Multiplex exon amplification using gene-specific/zip code primers.
PCR 2
Many exons
2. Taq polymerase, amplify all primary products using zip-code primers.
3. Dilute 1/20.
LDR 1
4. Taq Ligase, hot start. Allele specific multiplex linear amplification.
5a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
5b. Capture on addressable array and fluorescent quantification.
Normal Allele
Mutant Allele
Normal Allele
Mutant Allele

FIG. 1

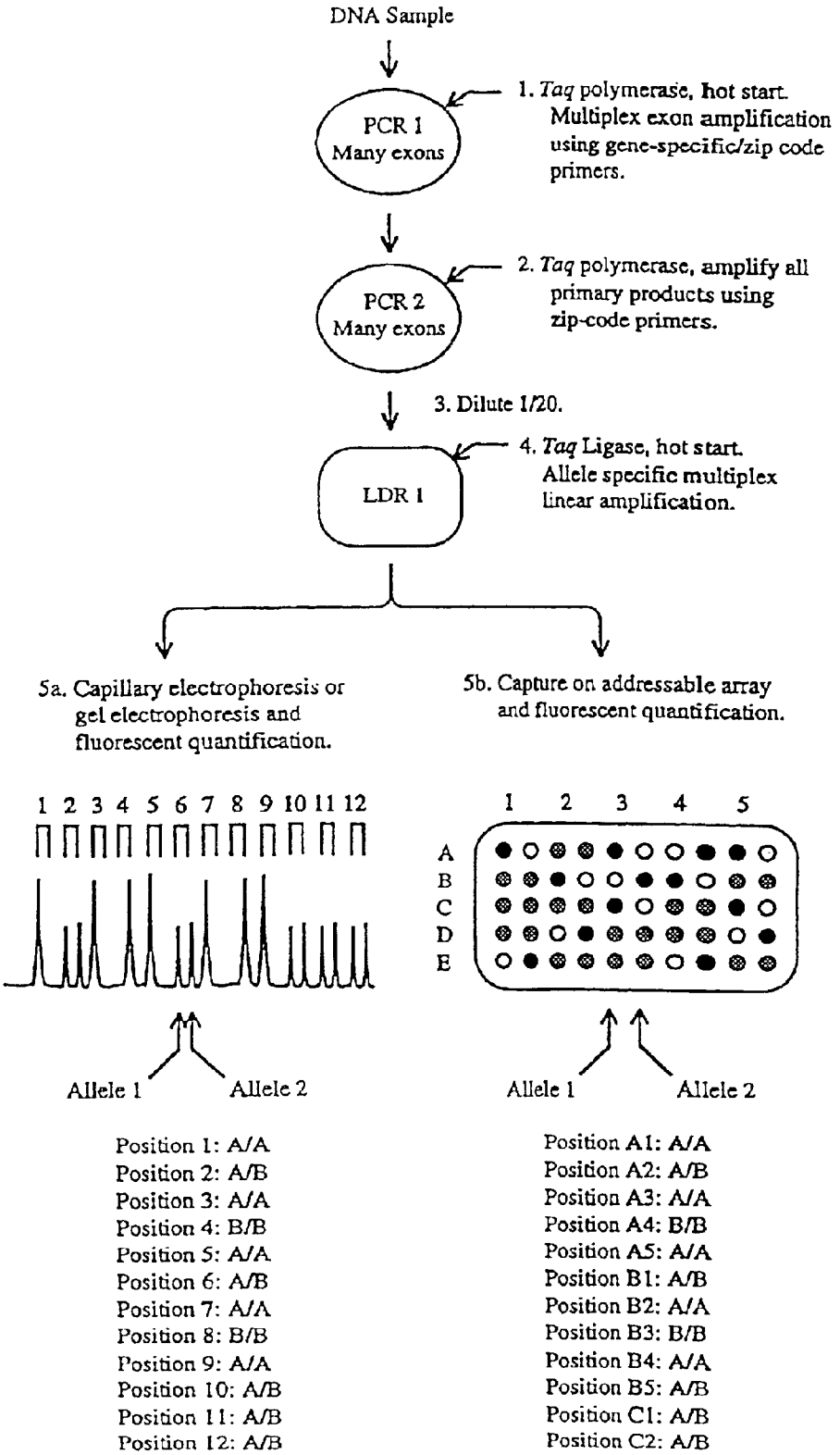

DNA Sample
PCR 1
Many exons
1. Taq polymerase, hot start. Multiplex exon amplification using gene-specific/zip code primers.
PCR 2
Many exons
2. Taq polymerase, amplify all primary products using zip-code primers.
3. Dilute 1/20.
LDR 1
4. Taq Ligase, hot start. Allele specific multiplex linear amplification.
5a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
5b. Capture on addressable array and fluorescent quantification.
1 2 3 4 5 6 7 8 9 10 11 12
Allele 1
Allele 2
1 2 3 4 5
A
B
C
D
E
Allele 1
Allele 2
Position 1: A/A
Position 2: A/B
Position 3: A/A
Position 4: B/B
Position 5: A/A
Position 6: A/B
Position 7: A/A
Position 8: B/B
Position 9: A/A
Position 10: A/B
Position 11: A/B
Position 12: A/B
Position A1: A/A
Position A2: A/B
Position A3: A/A
Position A4: B/B
Position A5: A/A
Position B1: A/B
Position B2: A/A
Position B3: B/B
Position B4: A/A
Position B5: A/B
Position C1: A/B
Position C2: A/B

FIG. 2

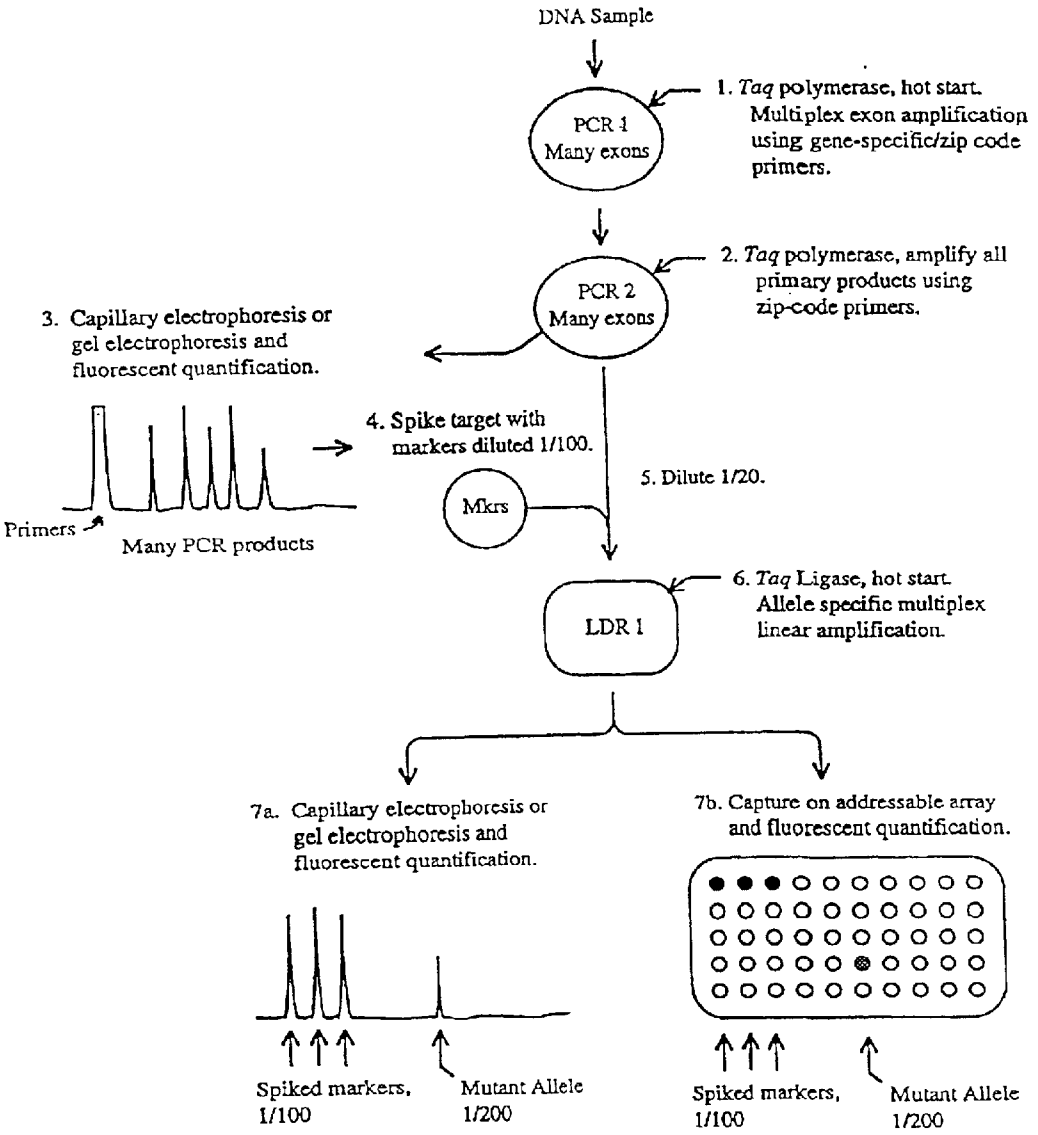

DNA Sample
PCR 1
Many exons
1. Taq polymerase, hot start. Multiplex exon amplification using gene-specific/zip code primers.
PCR 2
Many exons
2. Taq polymerase, amplify all primary products using zip-code primers.
3. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Primers
Many PCR products
4. Spike target with markers diluted 1/100.
Mkrs
5. Dilute 1/20.
LDR 1
6. Taq Ligase, hot start. Allele specific multiplex linear amplification.
7a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200
7b. Capture on addressable array and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200

FIG. 3

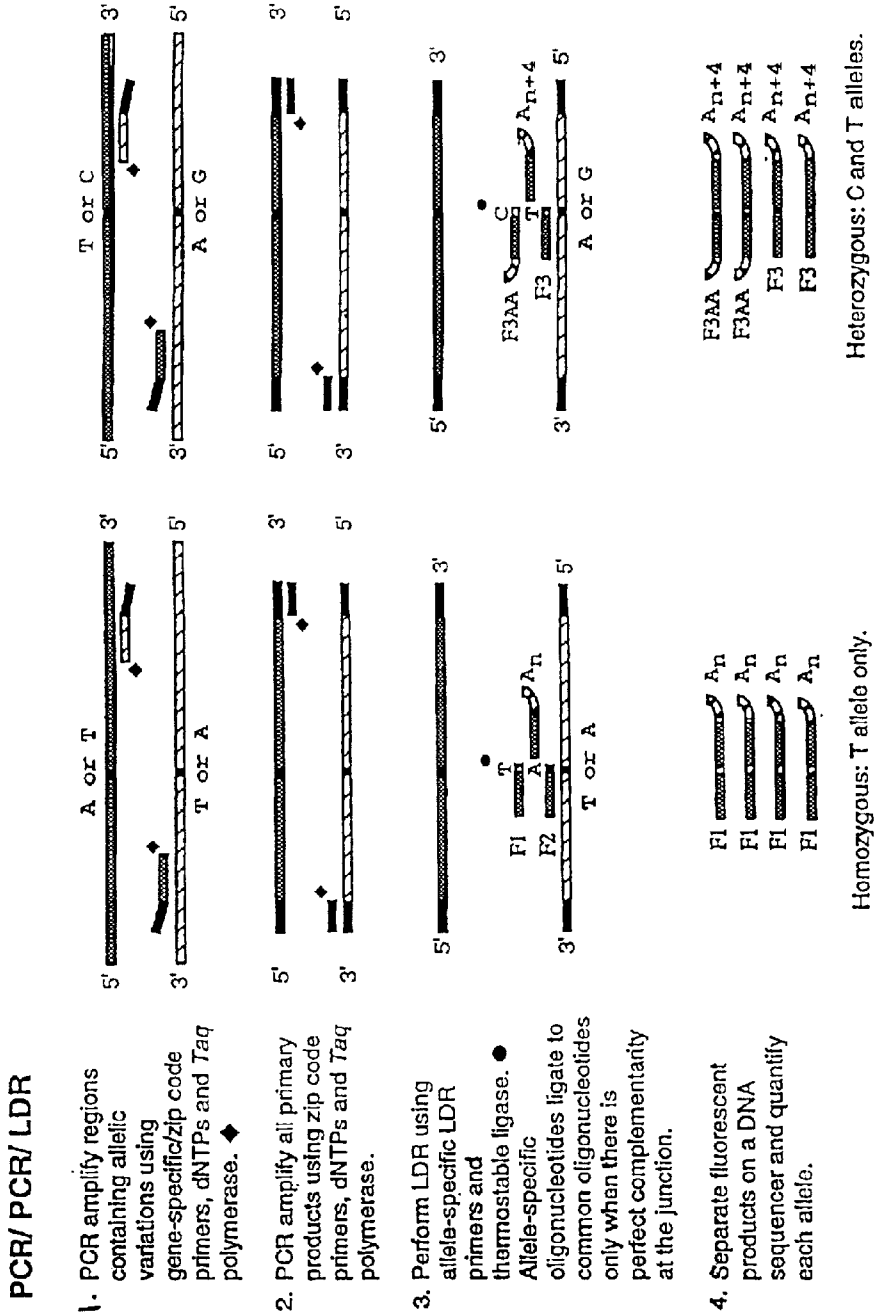

PCR/ PCR/ LDR
1. PCR amplify regions containing allelic variations using gene-specific/zip code primers, dNTPs and Taq polymerase. ◆
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase.
3. Perform LDR using allele-specific LDR primers and thermostable ligase. ● Allele-specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
4. Separate fluorescent products on a DNA sequencer and quantify each allele.
A or T
T or A
T or C
A or G
F1
F2
F3
F3AA
Homozygous: T allele only.
Heterozygous: C and T alleles.

FIG. 4

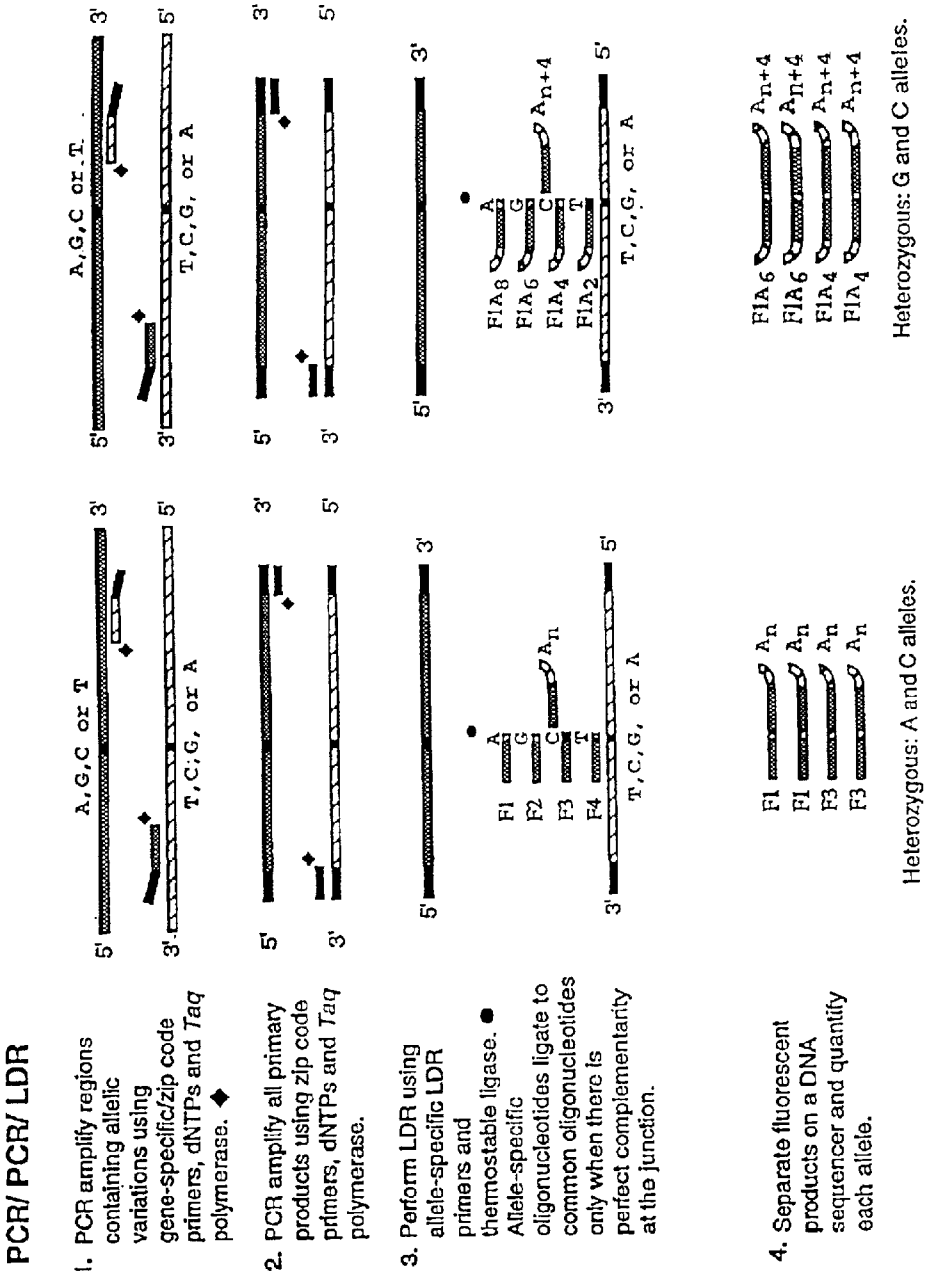

PCR/ PCR/ LDR
1. PCR amplify regions containing allelic variations using gene-specific/zip code primers, dNTPs and Taq polymerase.
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase.
3. Perform LDR using allele-specific LDR primers and thermostable ligase.
Allele-specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
4. Separate fluorescent products on a DNA sequencer and quantify each allele.
A,G,C or T
T,C;G, or A
T,C,G, or A
Heterozygous: A and C alleles.
Heterozygous: G and C alleles.

FIG. 5

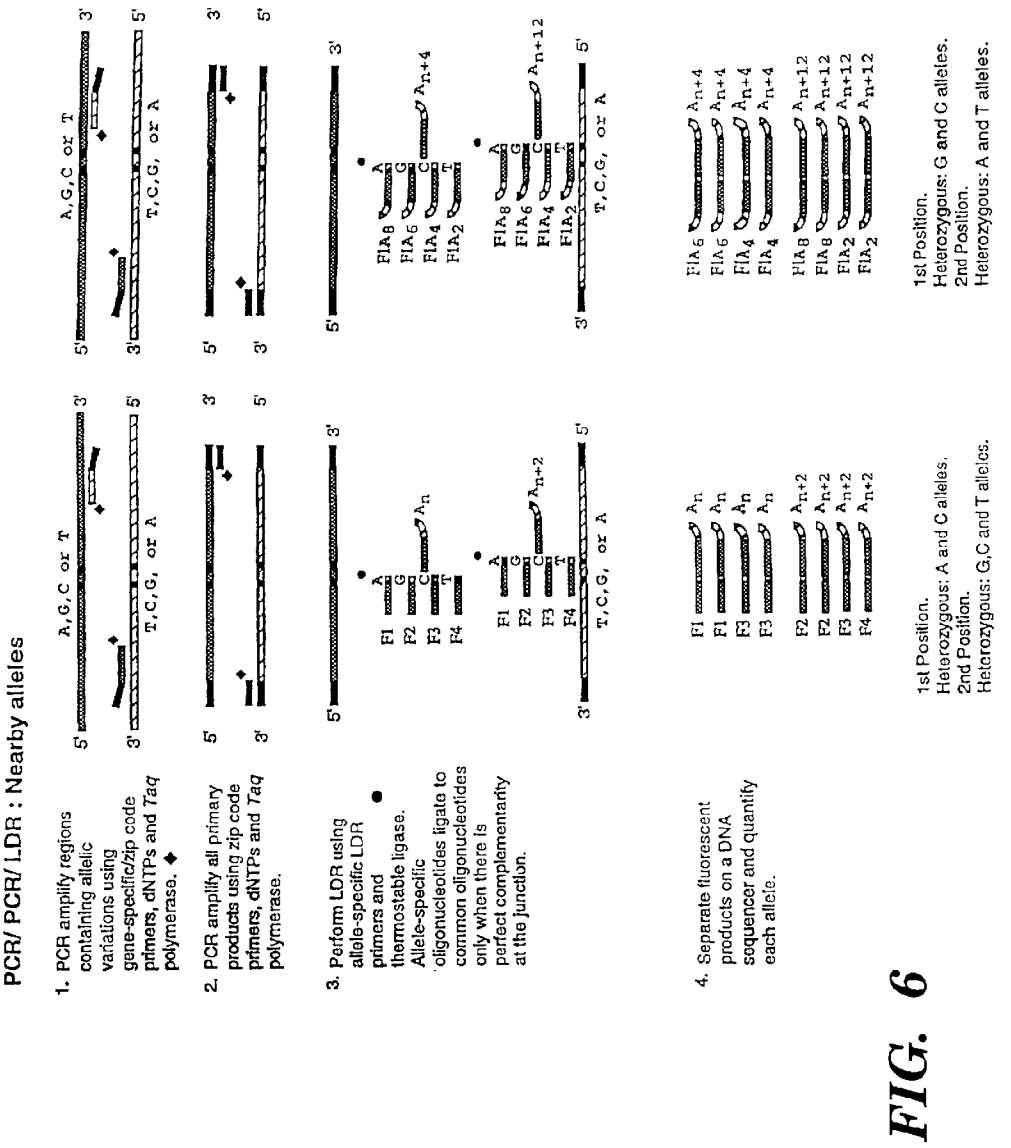

PCR/ PCR/ LDR : Nearby alleles
1. PCR amplify regions containing allelic variations using gene-specific/zip code primers, dNTPs and Taq polymerase.
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase.
3. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele-specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
4. Separate fluorescent products on a DNA sequencer and quantify each allele.
A,G,C or T
T,C,G, or A
F1
F2
F3
F4
1st Position.
Heterozygous: A and C alleles.
2nd Position.
Heterozygous: G,C and T alleles.
1st Position.
Heterozygous: G and C alleles.
2nd Position.
Heterozygous: A and T alleles.

FIG. 6

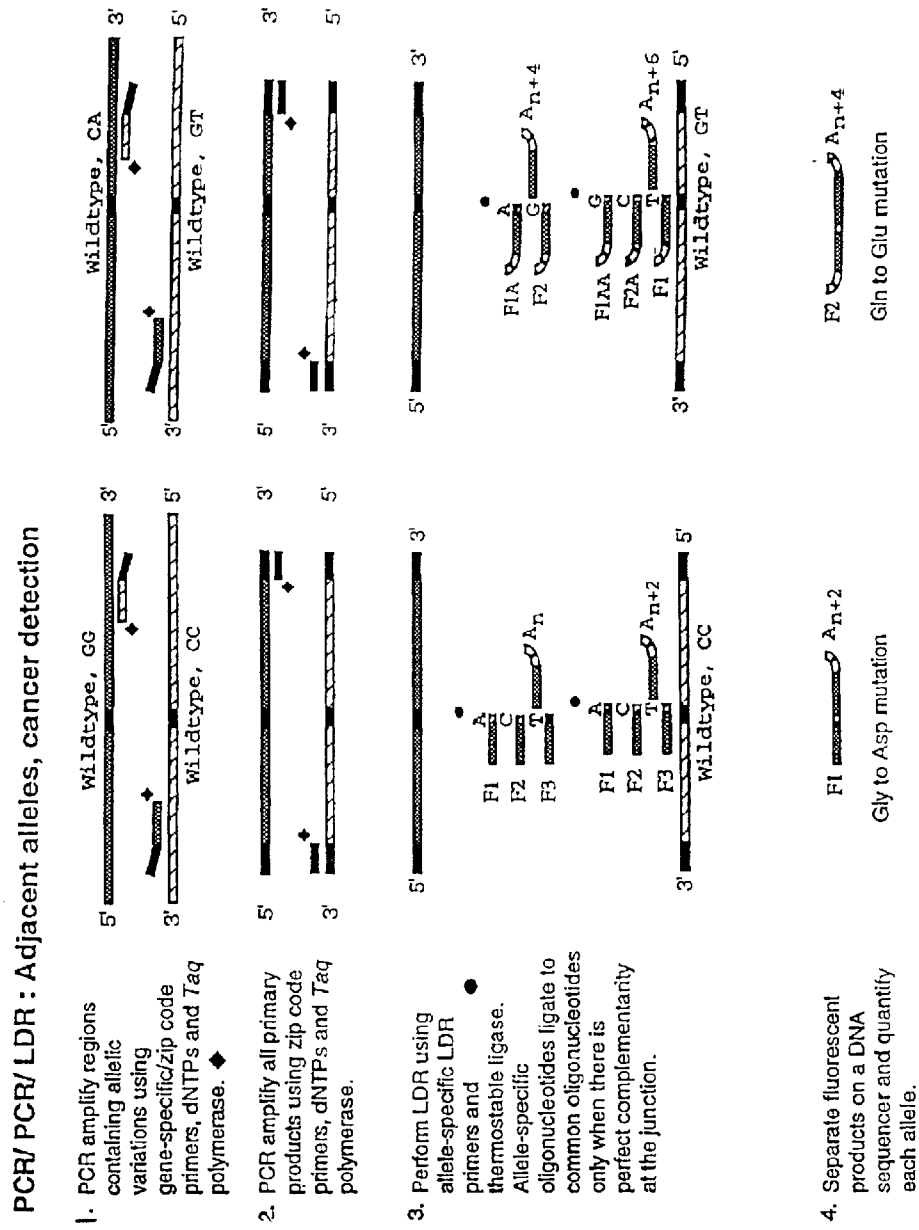

PCR/ PCR/ LDR : Adjacent alleles, cancer detection
1. PCR amplify regions containing allelic variations using gene-specific/zip code primers, dNTPs and Taq polymerase. ◆
Wildtype, GG
Wildtype, CC
Wildtype, CA
Wildtype, GT
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase.
3. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele-specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
F1
F2
F3
F1A
F2A
4. Separate fluorescent products on a DNA sequencer and quantify each allele.
Gly to Asp mutation
Gln to Glu mutation

FIG. 7

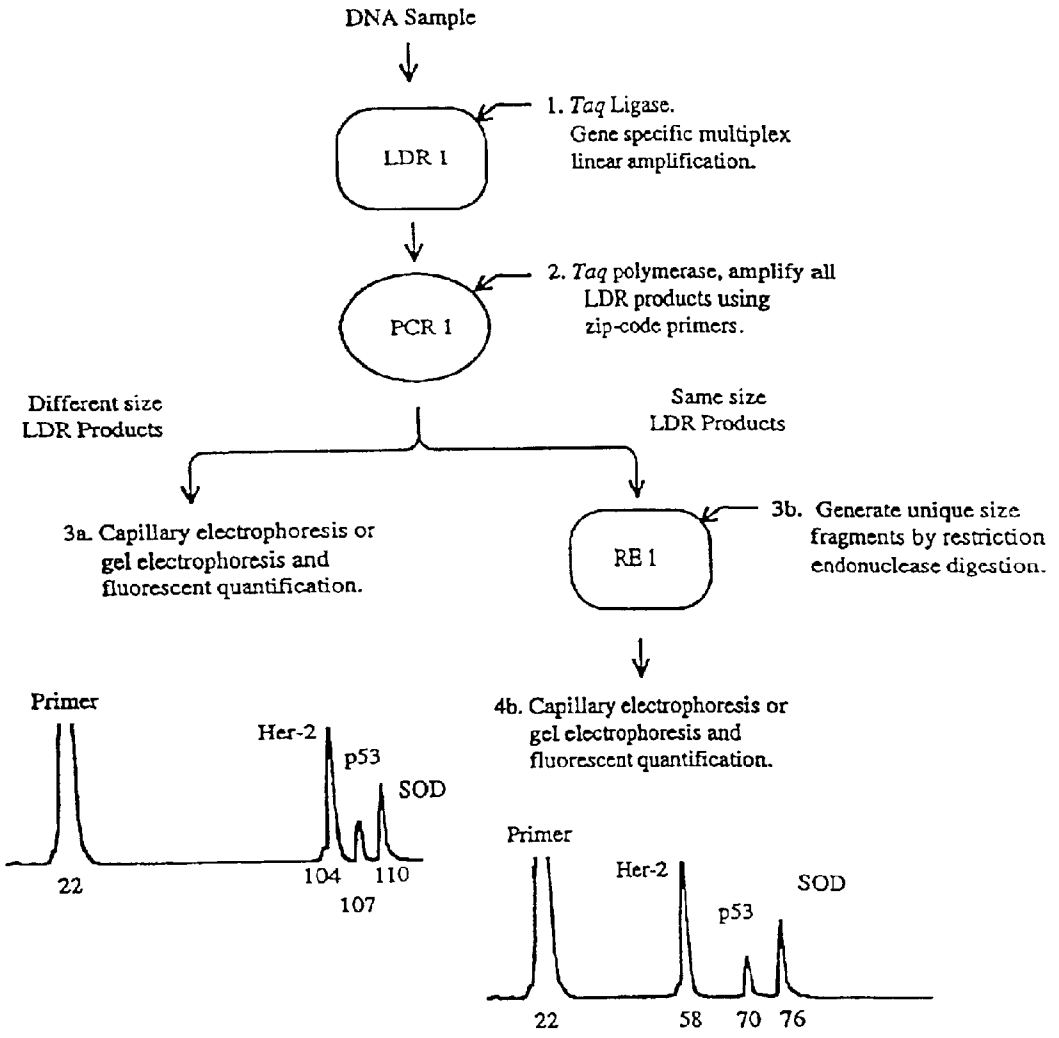

DNA Sample
LDR 1
1. Taq Ligase. Gene specific multiplex linear amplification.
PCR 1
2. Taq polymerase, amplify all LDR products using zip-code primers.
Different size LDR Products
Same size LDR Products
3a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
RE 1
3b. Generate unique size fragments by restriction endonuclease digestion.
4b. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Primer
Her-2
p53
SOD
22
104
107
110
Primer
Her-2
p53
SOD
22
58
70
76

FIG. 8

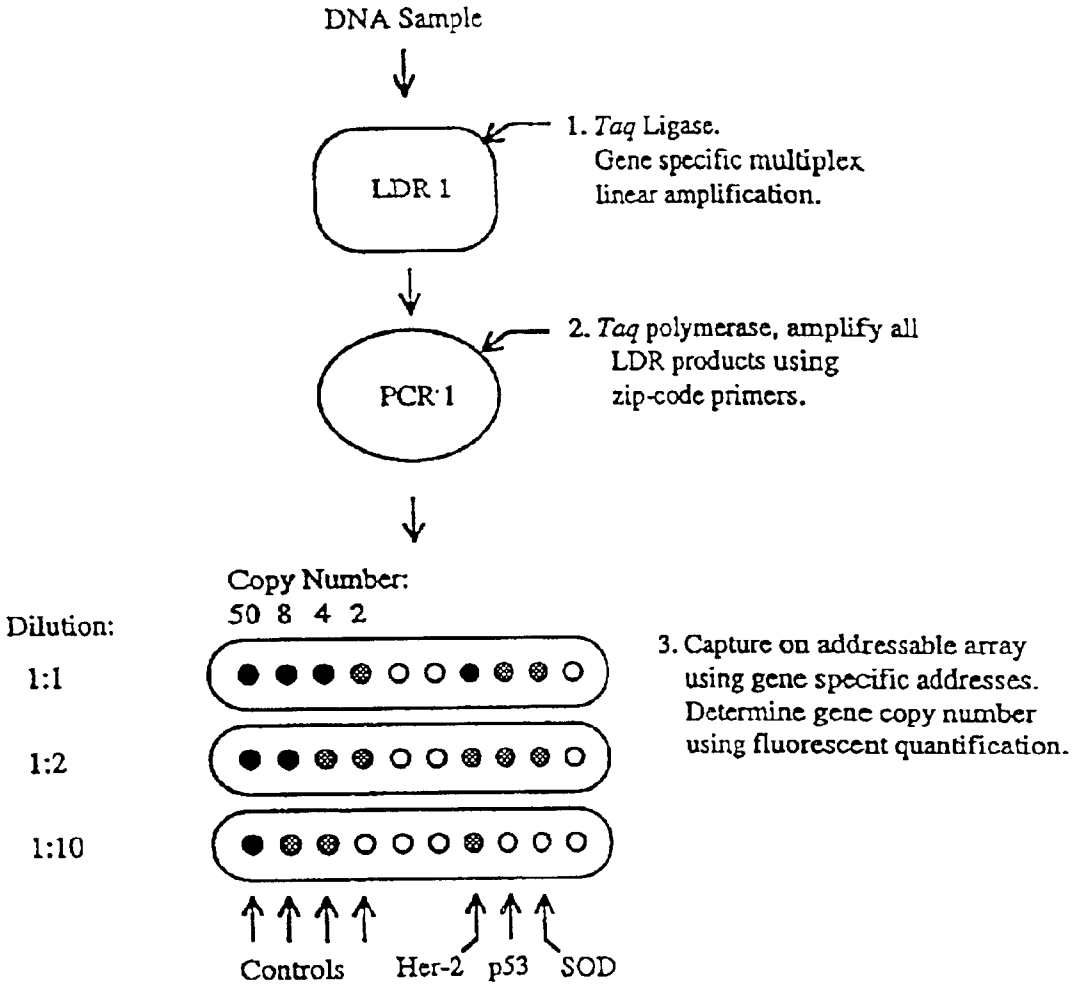

DNA Sample
LDR 1
1. Taq Ligase. Gene specific multiplex linear amplification.
PCR 1
2. Taq polymerase, amplify all LDR products using zip-code primers.
Copy Number:
50 8 4 2
Dilution:
1:1
1:2
1:10
3. Capture on addressable array using gene specific addresses. Determine gene copy number using fluorescent quantification.
Controls
Her-2
p53
SOD

FIG. 9

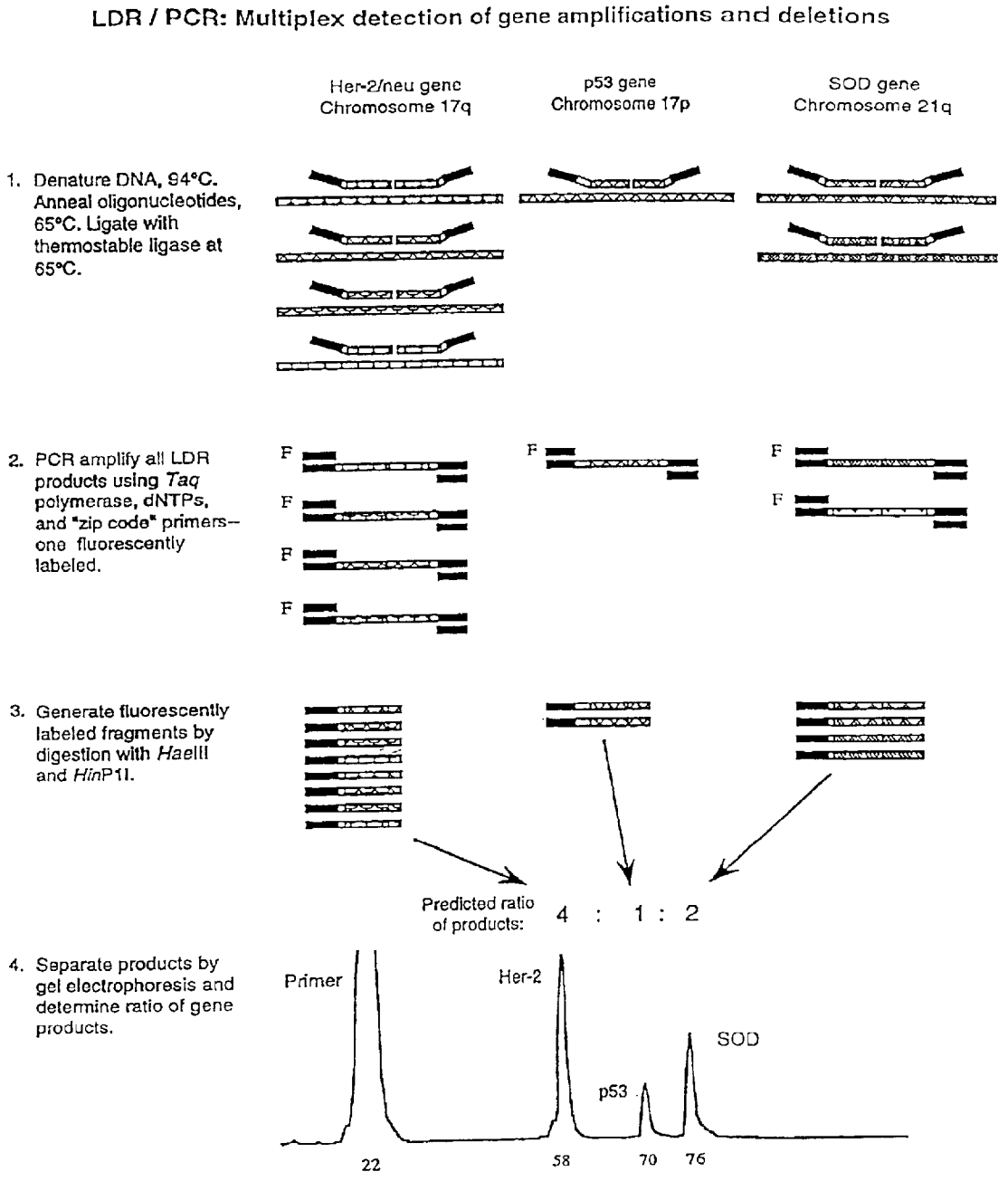

LDR / PCR: Multiplex detection of gene amplifications and deletions
Her-2/neu gene Chromosome 17q
p53 gene Chromosome 17p
SOD gene Chromosome 21q
1. Denature DNA, 94°C. Anneal oligonucleotides, 65°C. Ligate with thermostable ligase at 65°C.
2. PCR amplify all LDR products using Taq polymerase, dNTPs, and "zip code" primers–one fluorescently labeled.
F
3. Generate fluorescently labeled fragments by digestion with HaeIII and HinP1I.
Predicted ratio of products: 4 : 1 : 2
4. Separate products by gel electrophoresis and determine ratio of gene products.
Primer
Her-2
p53
SOD
22
58
70
76

FIG. 10

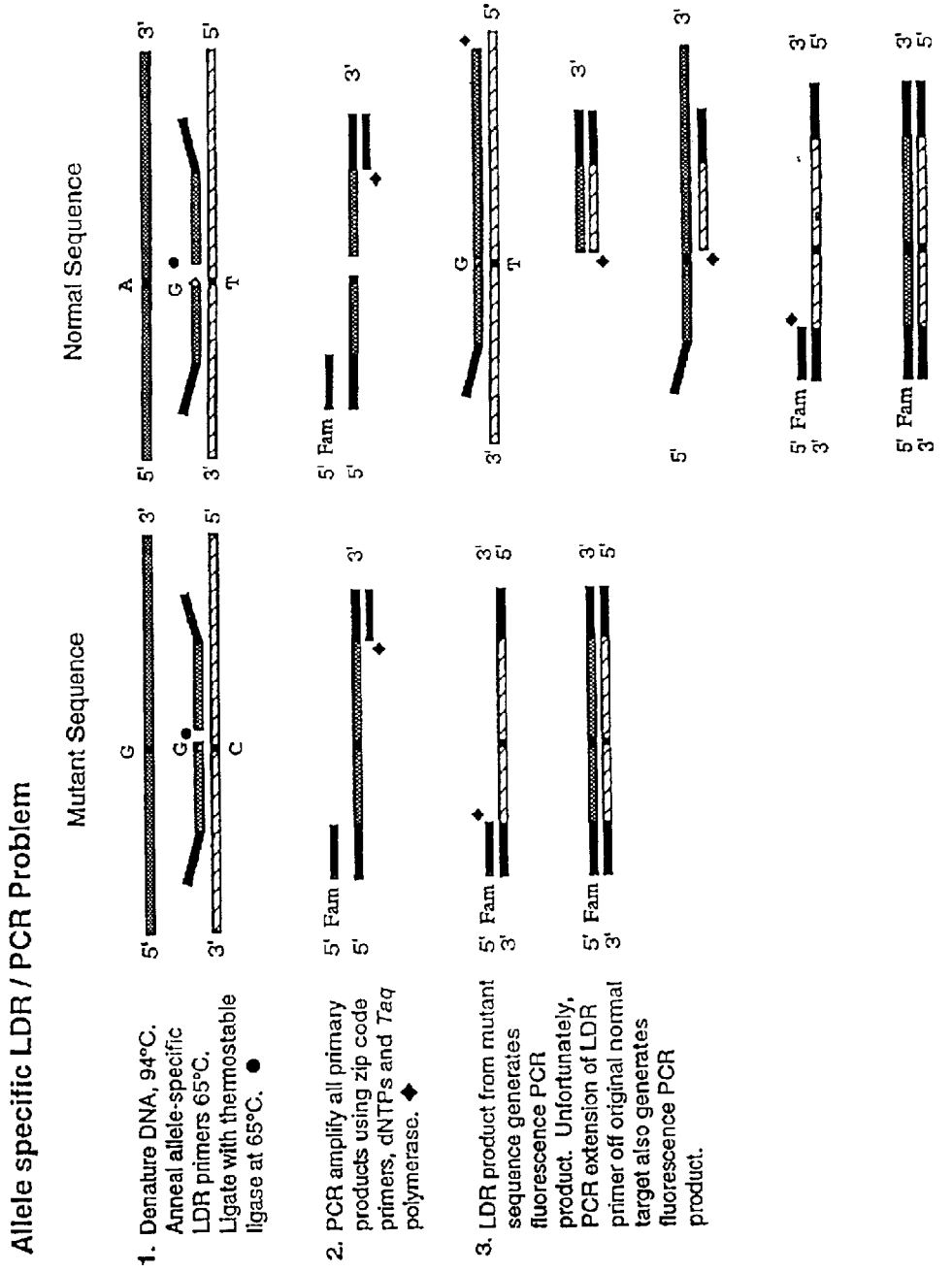

Allele specific LDR / PCR Problem
Mutant Sequence
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific LDR primers 65°C. Ligate with thermostable ligase at 65°C. ●
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
3. LDR product from mutant sequence generates fluorescence PCR product. Unfortunately, PCR extension of LDR primer off original normal target also generates fluorescence PCR product.
5' Fam
3'
5'

FIG. 11

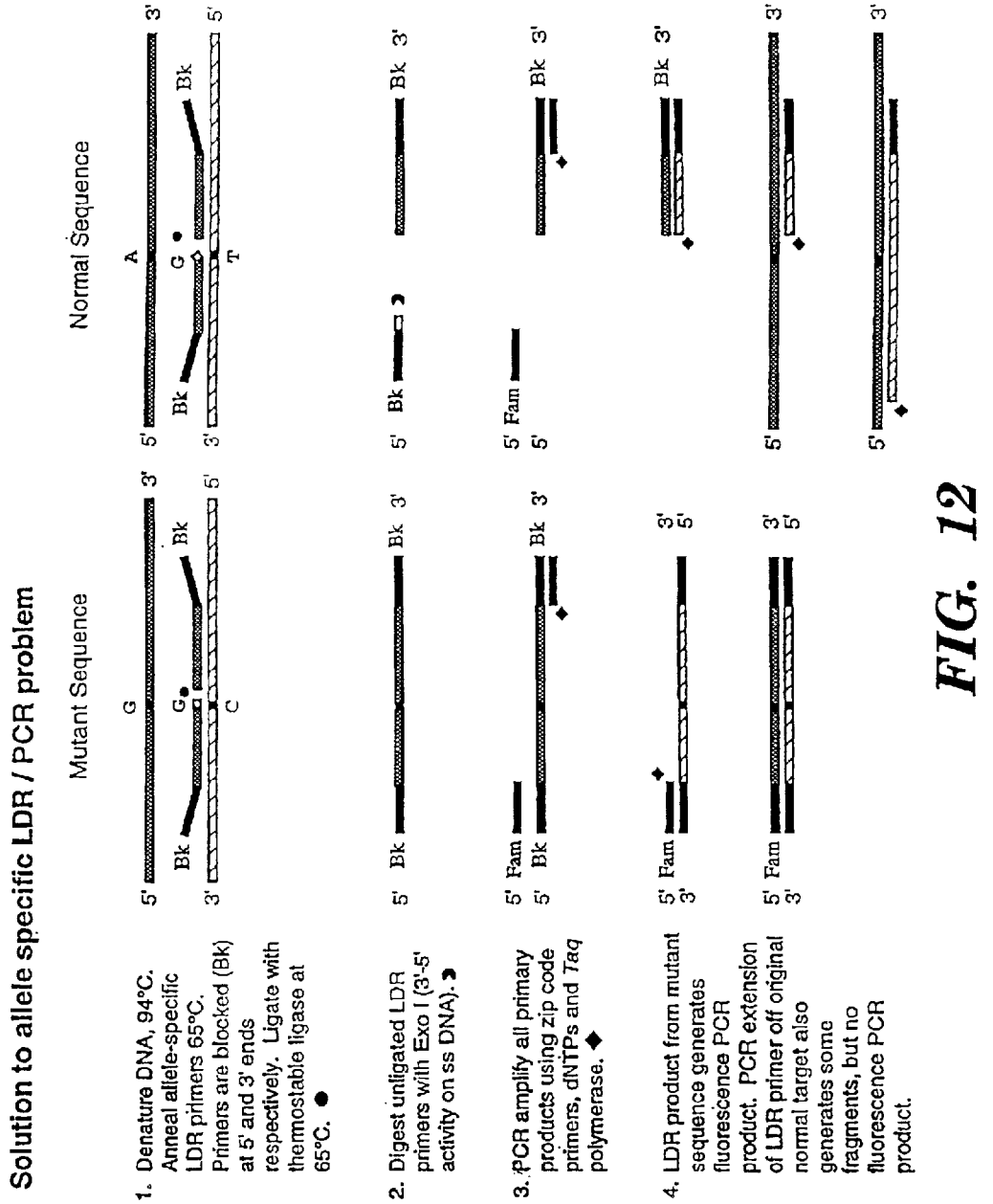

Solution to allele specific LDR / PCR problem
Mutant Sequence
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific LDR primers 65°C. Primers are blocked (Bk) at 5' and 3' ends respectively. Ligate with thermostable ligase at 65°C. ●
2. Digest unligated LDR primers with Exo I (3'-5' activity on ss DNA). ◗
3. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
4. LDR product from mutant sequence generates fluorescence PCR product. PCR extension of LDR primer off original normal target also generates some fragments, but no fluorescence PCR product.
5' 3' Bk G A T C Fam

FIG. 12

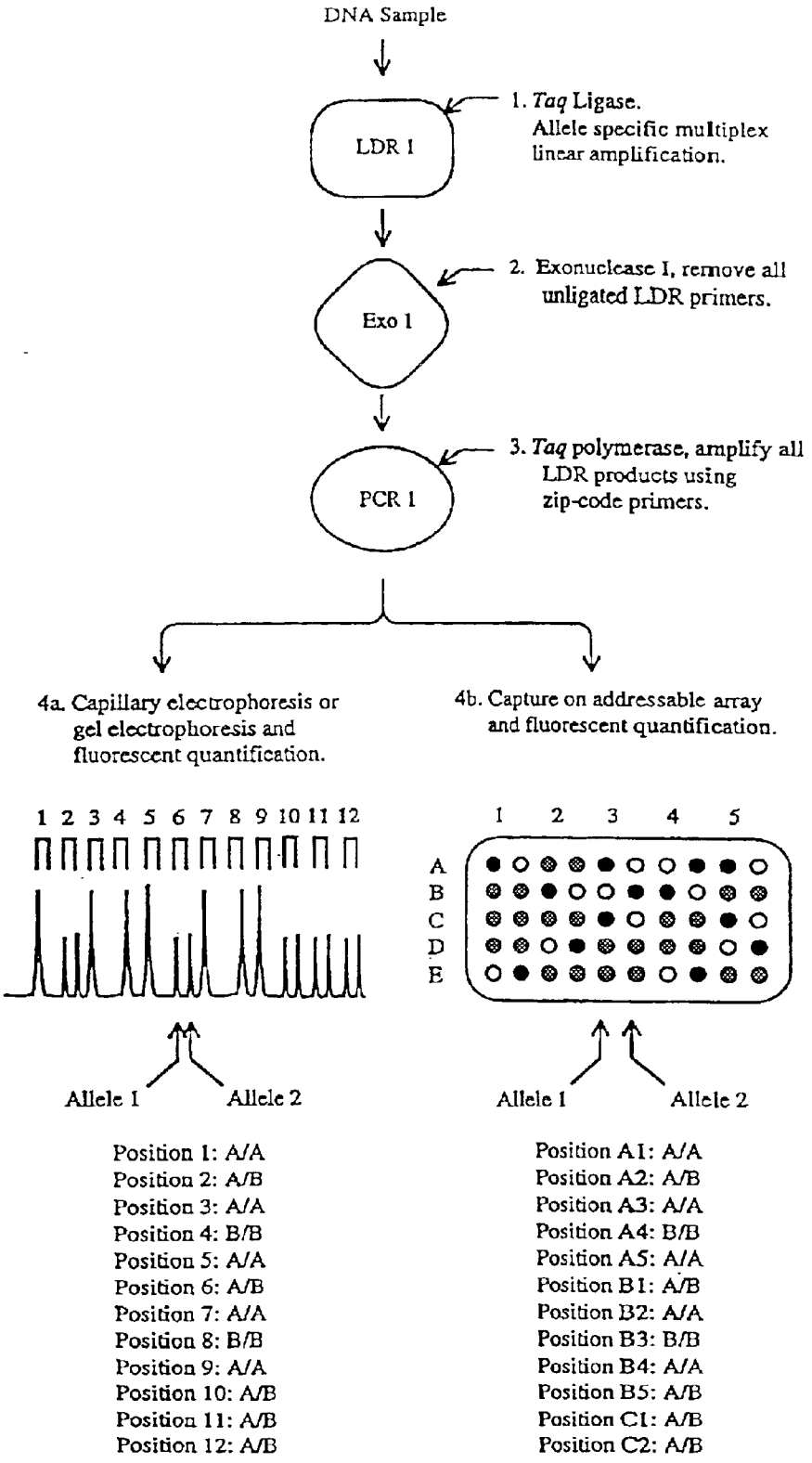

DNA Sample
LDR 1
1. Taq Ligase. Allele specific multiplex linear amplification.
Exo 1
2. Exonuclease I, remove all unligated LDR primers.
PCR 1
3. Taq polymerase, amplify all LDR products using zip-code primers.
4a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
4b. Capture on addressable array and fluorescent quantification.
1 2 3 4 5 6 7 8 9 10 11 12
Allele 1
Allele 2
Position 1: A/A
Position 2: A/B
Position 3: A/A
Position 4: B/B
Position 5: A/A
Position 6: A/B
Position 7: A/A
Position 8: B/B
Position 9: A/A
Position 10: A/B
Position 11: A/B
Position 12: A/B
1 2 3 4 5
A
B
C
D
E
Allele 1
Allele 2
Position A1: A/A
Position A2: A/B
Position A3: A/A
Position A4: B/B
Position A5: A/A
Position B1: A/B
Position B2: A/A
Position B3: B/B
Position B4: A/A
Position B5: A/B
Position C1: A/B
Position C2: A/B

FIG. 13

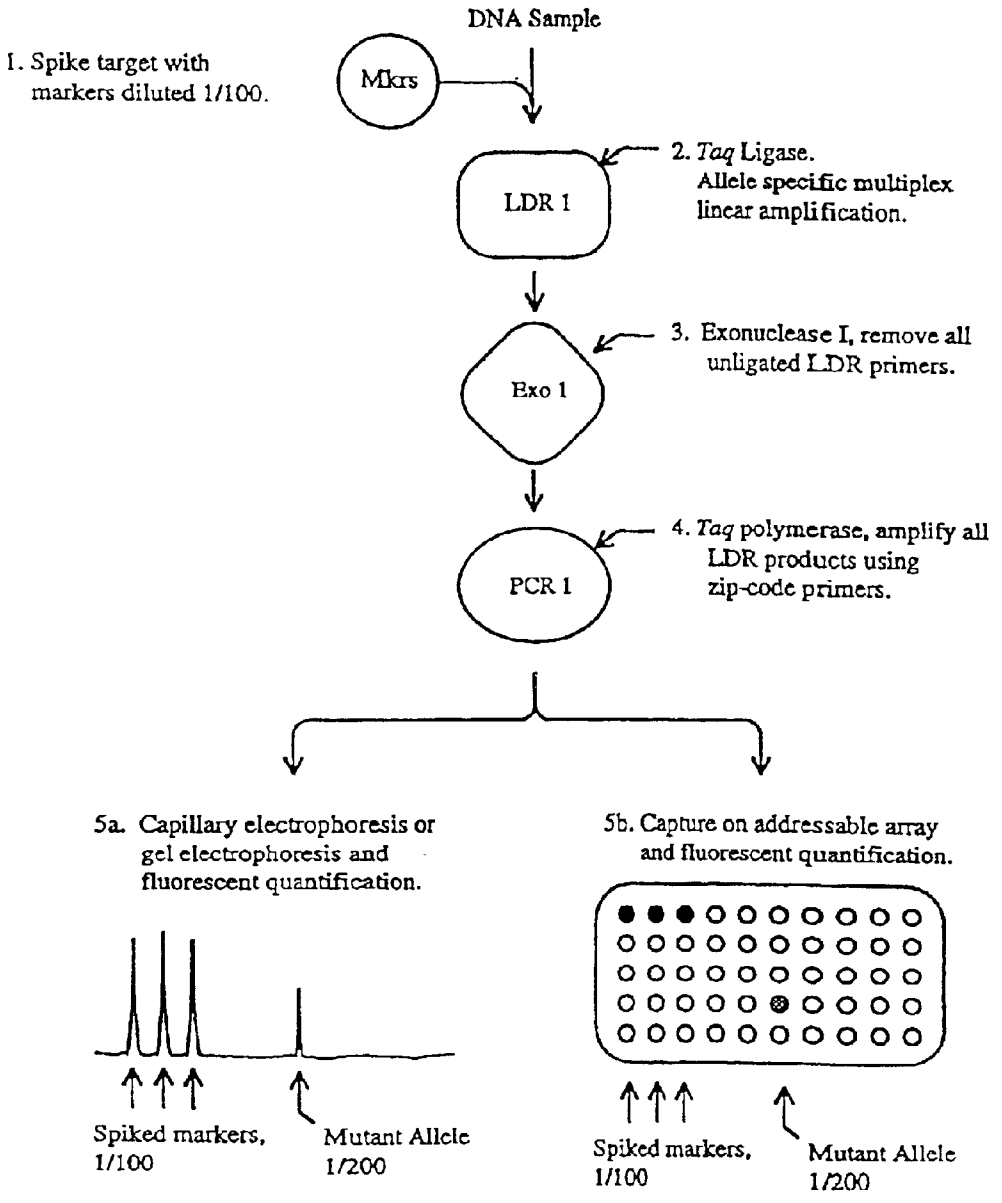

DNA Sample
1. Spike target with markers diluted 1/100.
Mkrs
LDR 1
2. Taq Ligase. Allele specific multiplex linear amplification.
Exo 1
3. Exonuclease I, remove all unligated LDR primers.
PCR 1
4. Taq polymerase, amplify all LDR products using zip-code primers.
5a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200
5b. Capture on addressable array and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200

FIG. 14

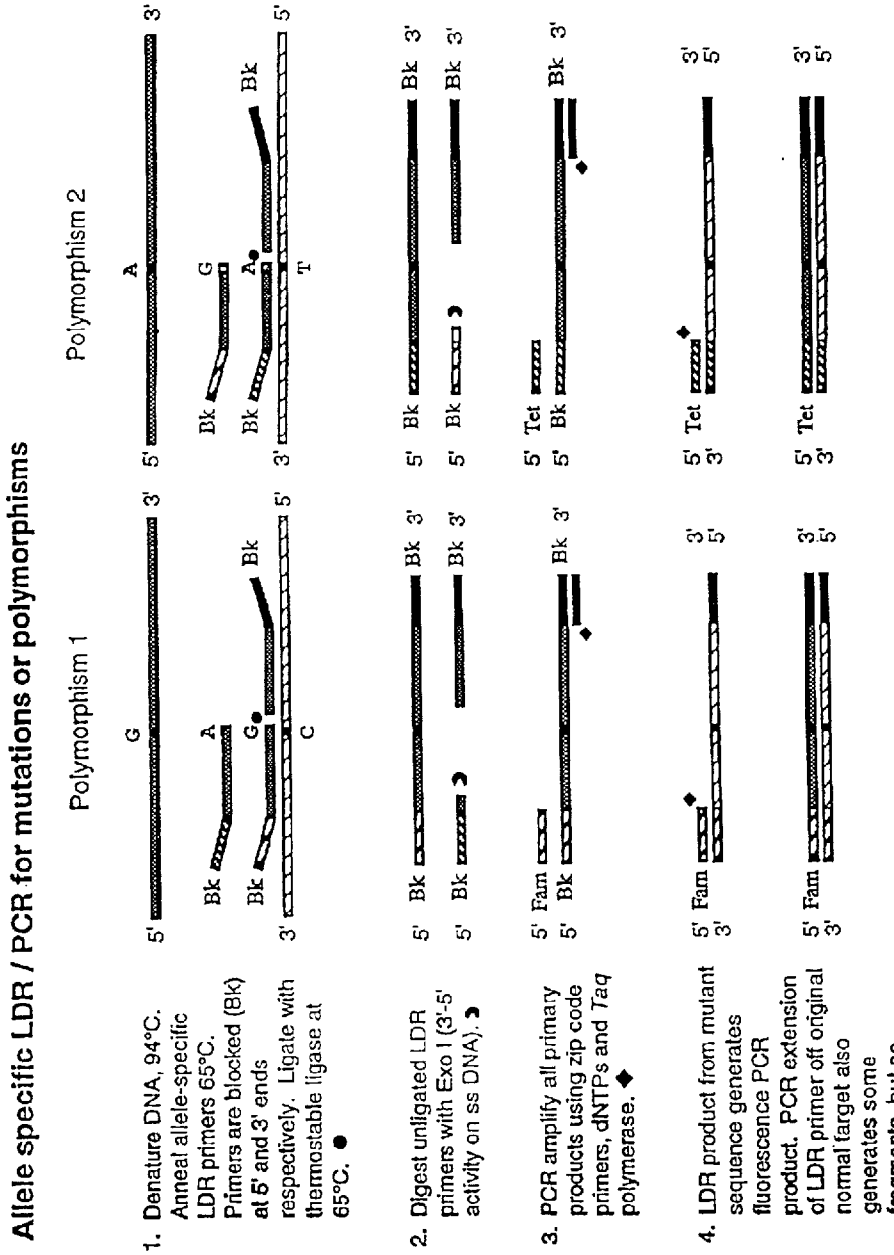

Allele specific LDR / PCR for mutations or polymorphisms
Polymorphism 1
Polymorphism 2
1. Denature DNA, 94°C. Anneal allele-specific LDR primers 65°C. Primers are blocked (Bk) at 5' and 3' ends respectively. Ligate with thermostable ligase at 65°C. ●
2. Digest unligated LDR primers with Exo I (3'-5' activity on ss DNA). ❯
3. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
4. LDR product from mutant sequence generates fluorescence PCR product. PCR extension of LDR primer off original normal target also generates some fragments, but no fluorescence PCR product.
5' 3' G A T C Bk Fam Tet

FIG. 15

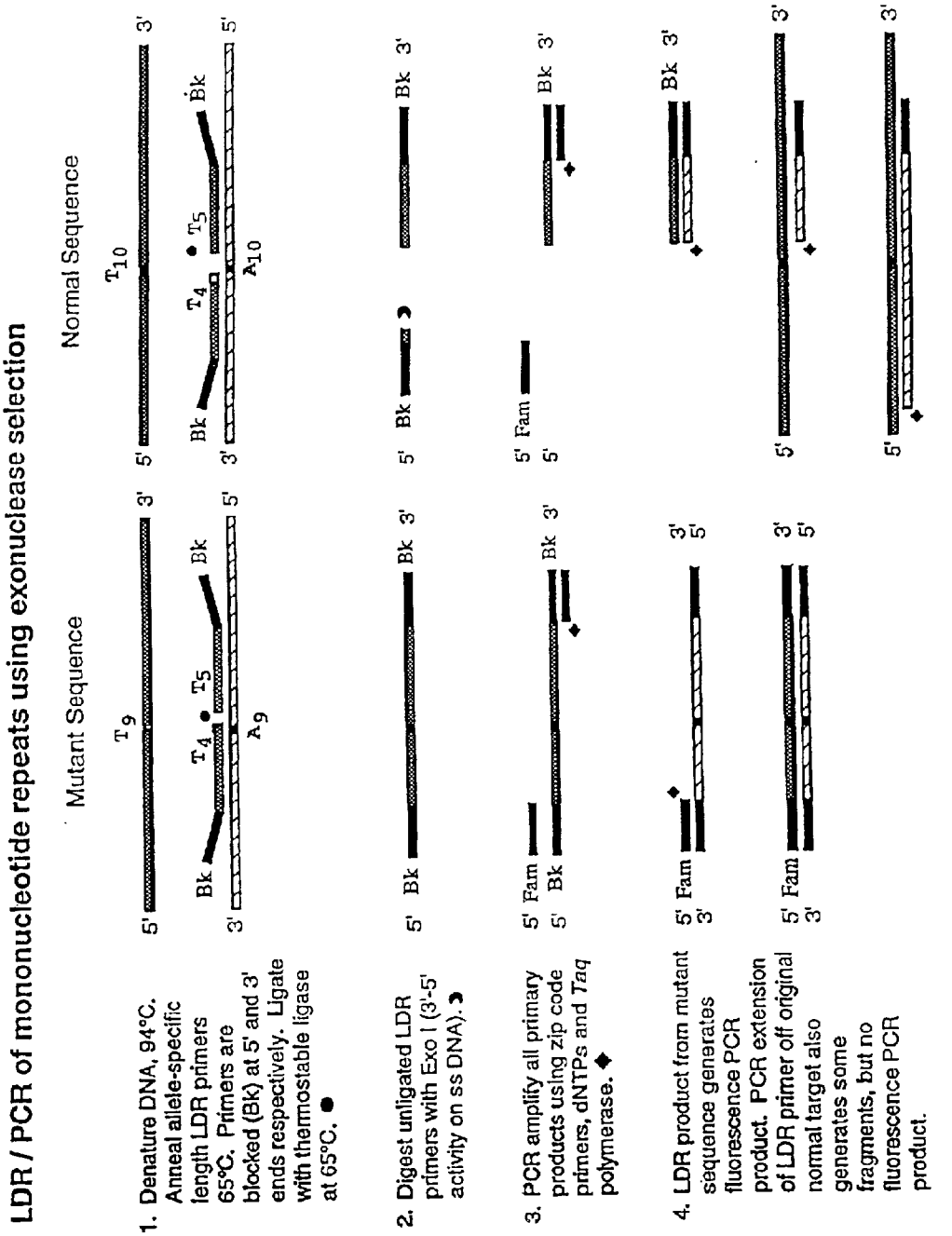

LDR / PCR of mononucleotide repeats using exonuclease selection
Mutant Sequence
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific length LDR primers 65°C. Primers are blocked (Bk) at 5' and 3' ends respectively. Ligate with thermostable ligase at 65°C. ●
2. Digest unligated LDR primers with Exo I (3'-5' activity on ss DNA). ❯
3. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
4. LDR product from mutant sequence generates fluorescence PCR product. PCR extension of LDR primer off original normal target also generates some fragments, but no fluorescence PCR product.
T9
T4
T5
A9
T10
A10
Bk
Fam
5'
3'

FIG. 16

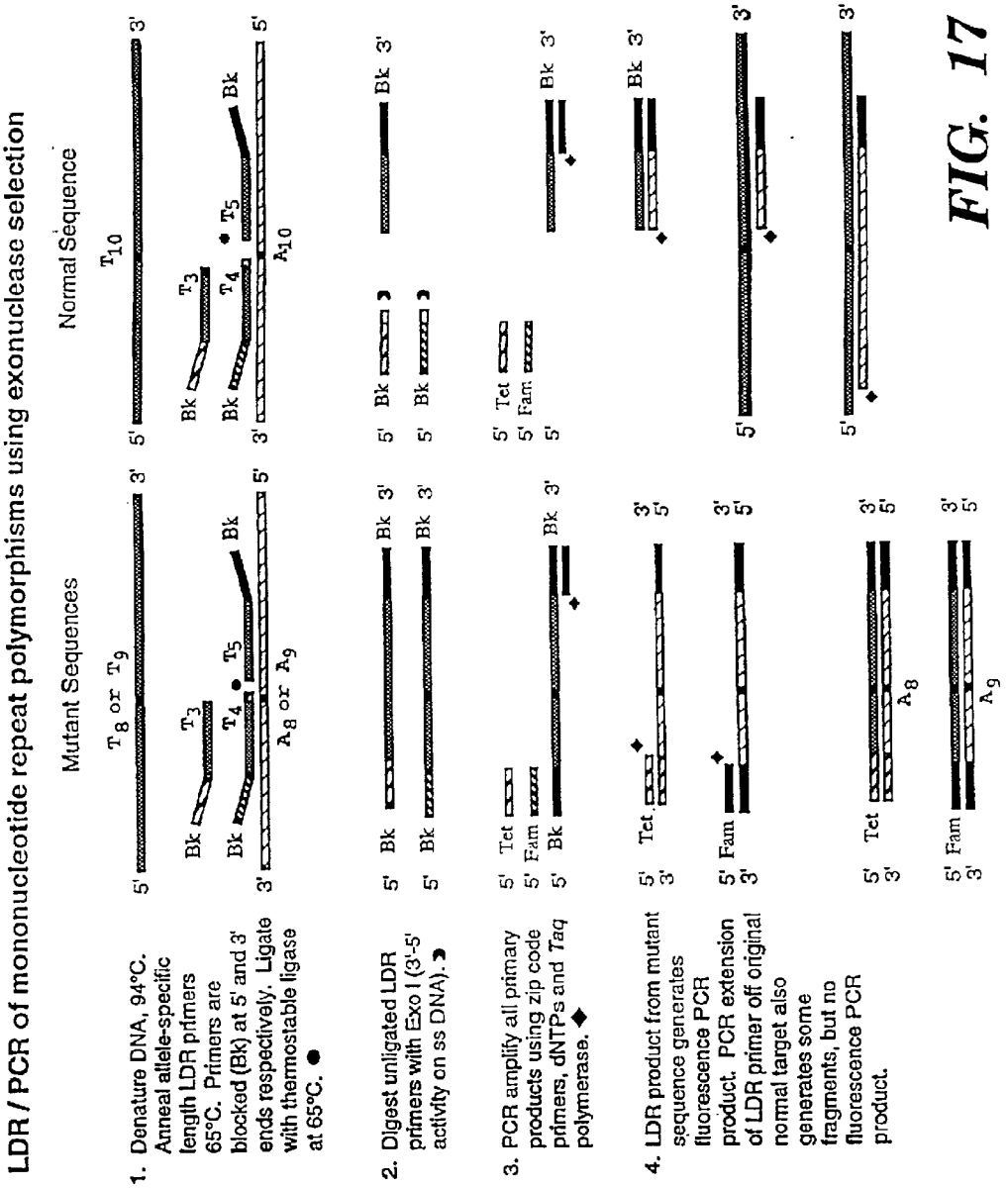

LDR / PCR of mononucleotide repeat polymorphisms using exonuclease selection
Mutant Sequences
Normal Sequence
T8 or T9
T10
A8 or A9
A10
1. Denature DNA, 94°C. Anneal allele-specific length LDR primers 65°C. Primers are blocked (Bk) at 5' and 3' ends respectively. Ligate with thermostable ligase at 65°C. ●
2. Digest unligated LDR primers with Exo I (3'-5' activity on ss DNA). ➤
3. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
4. LDR product from mutant sequence generates fluorescence PCR product. PCR extension of LDR primer off original normal target also generates some fragments, but no fluorescence PCR product.
Bk
Tet
Fam

FIG. 17

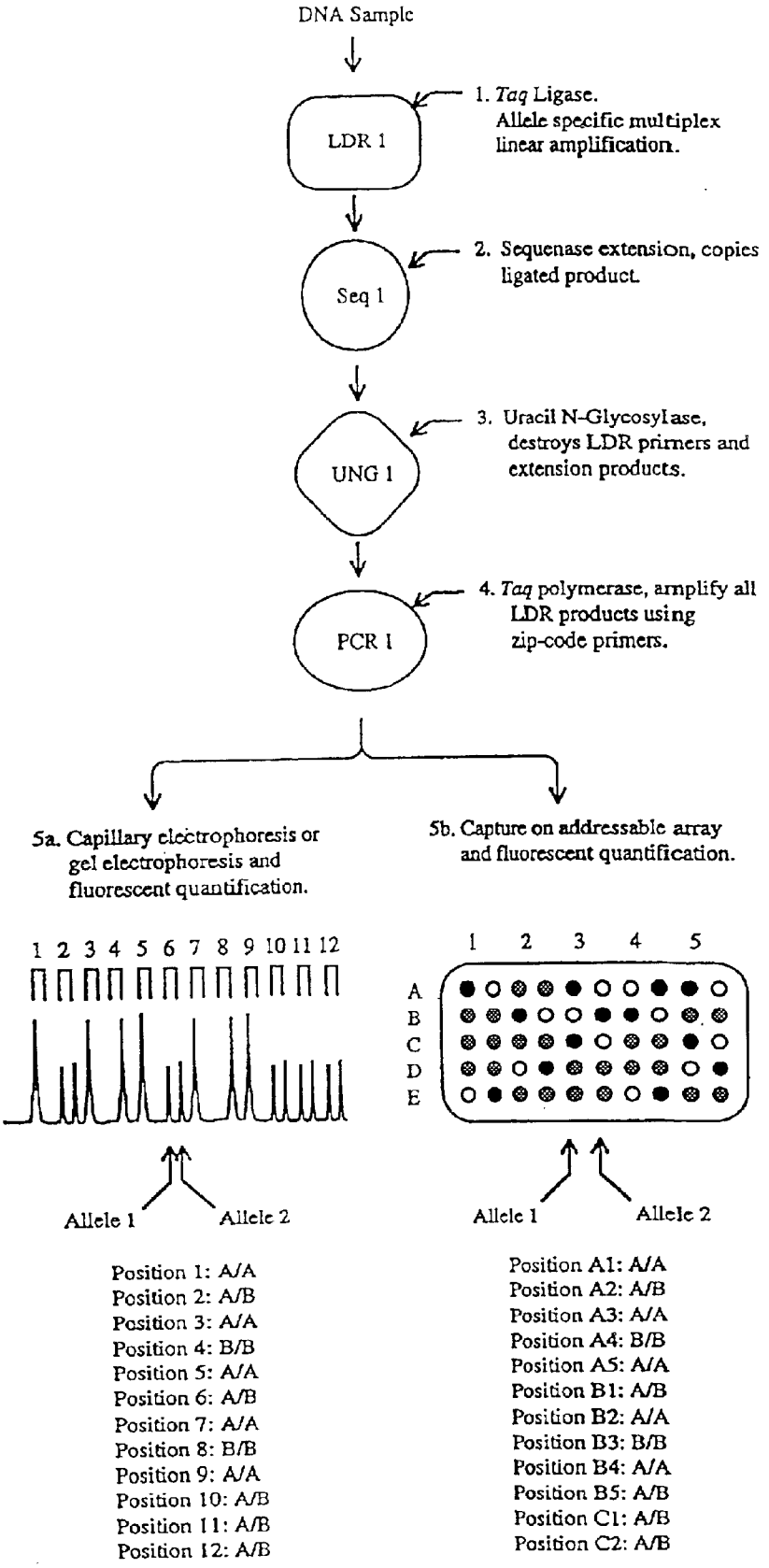

DNA Sample
LDR 1
1. Taq Ligase. Allele specific multiplex linear amplification.
Seq 1
2. Sequenase extension, copies ligated product.
UNG 1
3. Uracil N-Glycosylase, destroys LDR primers and extension products.
PCR 1
4. Taq polymerase, amplify all LDR products using zip-code primers.
5a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
5b. Capture on addressable array and fluorescent quantification.
1 2 3 4 5 6 7 8 9 10 11 12
Allele 1
Allele 2
Position 1: A/A
Position 2: A/B
Position 3: A/A
Position 4: B/B
Position 5: A/A
Position 6: A/B
Position 7: A/A
Position 8: B/B
Position 9: A/A
Position 10: A/B
Position 11: A/B
Position 12: A/B
1 2 3 4 5
A
B
C
D
E
Allele 1
Allele 2
Position A1: A/A
Position A2: A/B
Position A3: A/A
Position A4: B/B
Position A5: A/A
Position B1: A/B
Position B2: A/A
Position B3: B/B
Position B4: A/A
Position B5: A/B
Position C1: A/B
Position C2: A/B

FIG. 18

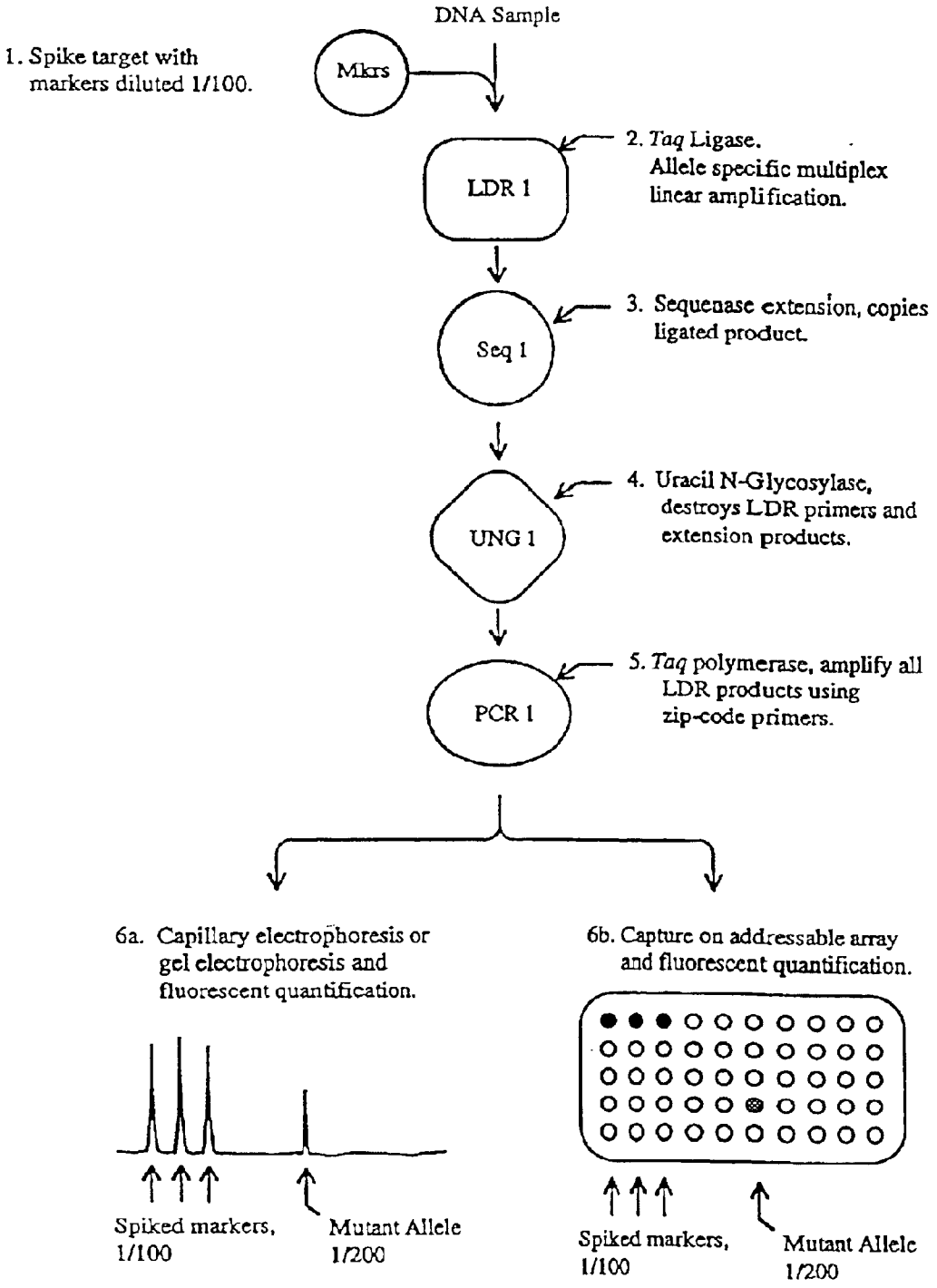

DNA Sample
1. Spike target with markers diluted 1/100.
Mkrs
LDR 1
2. Taq Ligase. Allele specific multiplex linear amplification.
Seq 1
3. Sequenase extension, copies ligated product.
UNG 1
4. Uracil N-Glycosylase, destroys LDR primers and extension products.
PCR 1
5. Taq polymerase, amplify all LDR products using zip-code primers.
6a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200
6b. Capture on addressable array and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200

FIG. 19

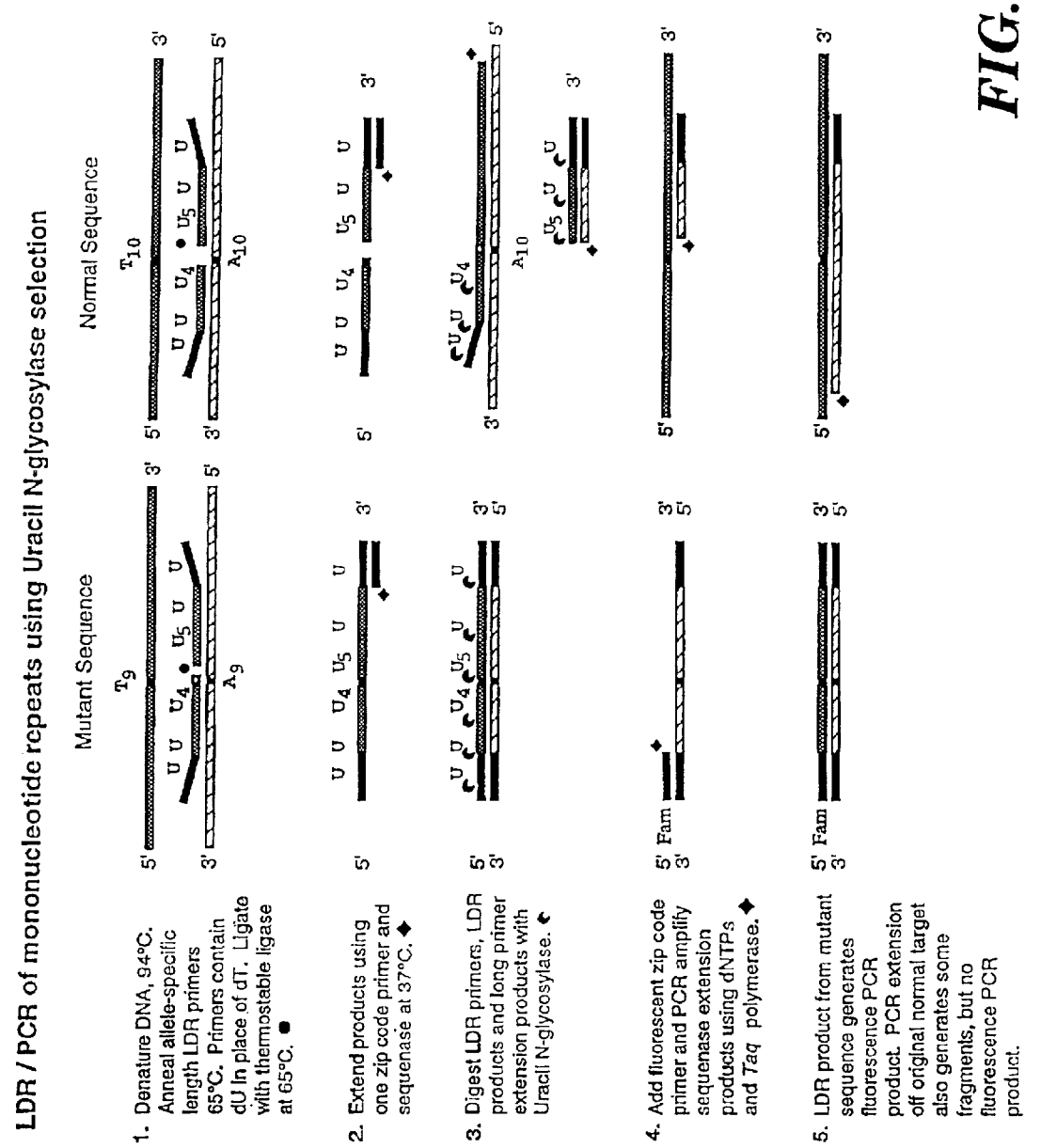

LDR / PCR of mononucleotide repeats using Uracil N-glycosylase selection
Mutant Sequence
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific length LDR primers 65°C. Primers contain dU In place of dT. Ligate with thermostable ligase at 65°C. ●
2. Extend products using one zip code primer and sequenase at 37°C. ✦
3. Digest LDR primers, LDR products and long primer extension products with Uracil N-glycosylase. ◐
4. Add fluorescent zip code primer and PCR amplify sequenase extension products using dNTPs and Taq polymerase. ✦
5. LDR product from mutant sequence generates fluorescence PCR product. PCR extension off original normal target also generates some fragments, but no fluorescence PCR product.
Fam

FIG. 20

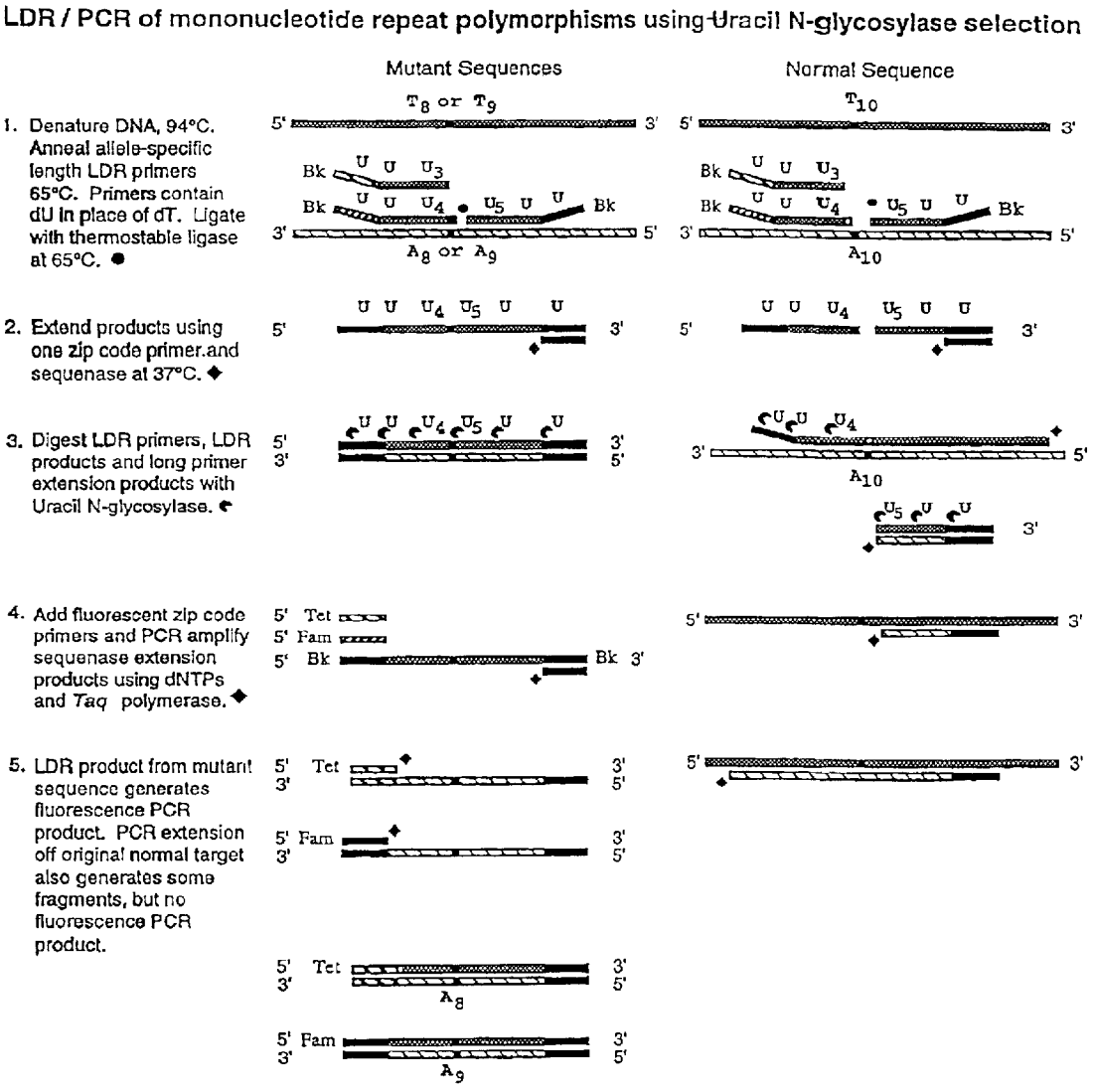

LDR / PCR of mononucleotide repeat polymorphisms using Uracil N-glycosylase selection
Mutant Sequences
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific length LDR primers 65°C. Primers contain dU in place of dT. Ligate with thermostable ligase at 65°C. ●
2. Extend products using one zip code primer.and sequenase at 37°C. ◆
3. Digest LDR primers, LDR products and long primer extension products with Uracil N-glycosylase. ◂
4. Add fluorescent zip code primers and PCR amplify sequenase extension products using dNTPs and Taq polymerase. ◆
5. LDR product from mutant sequence generates fluorescence PCR product. PCR extension off original normal target also generates some fragments, but no fluorescence PCR product.
T8 or T9
T10
A8 or A9
A10
Bk
Tet
Fam

FIG. 21

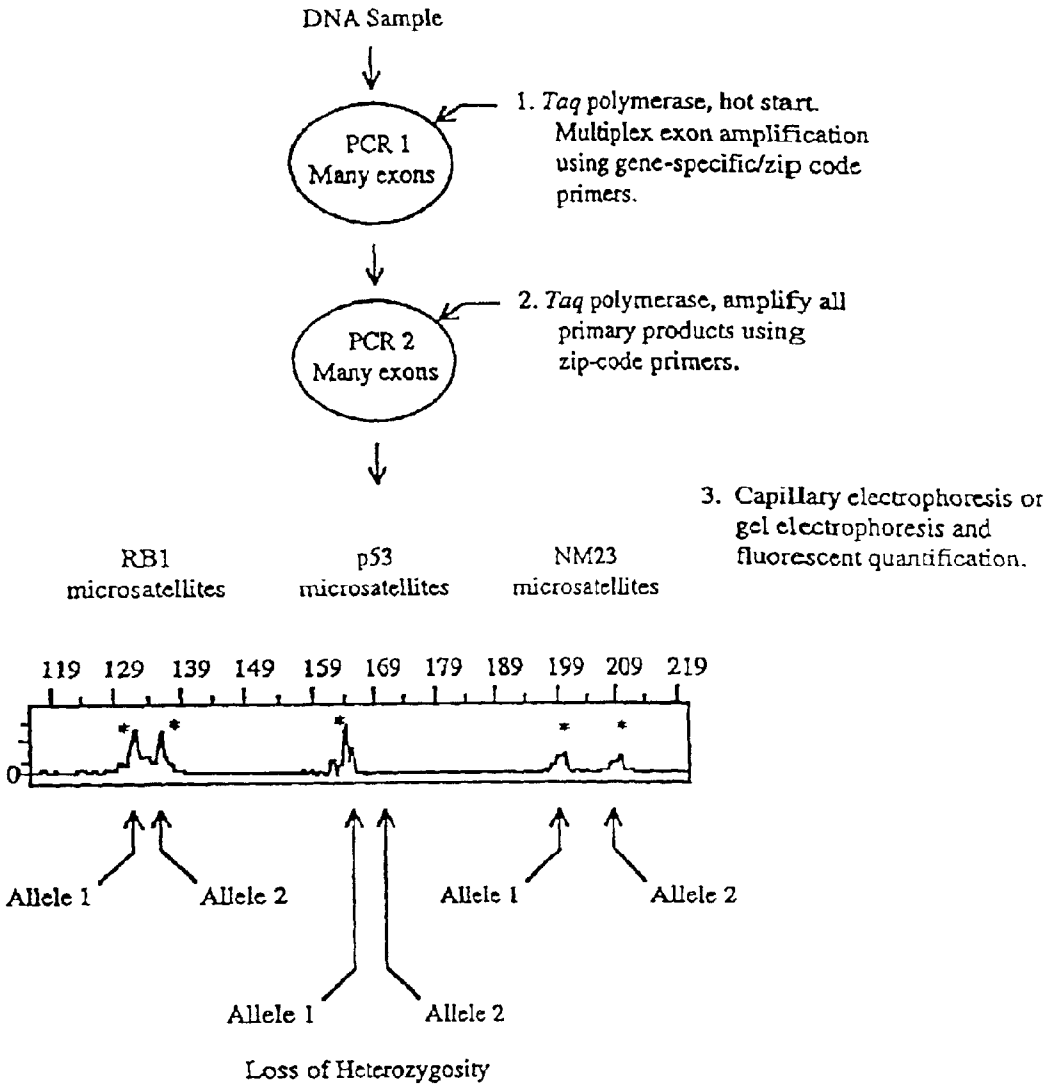

DNA Sample
PCR 1
Many exons
1. Taq polymerase, hot start. Multiplex exon amplification using gene-specific/zip code primers.
PCR 2
Many exons
2. Taq polymerase, amplify all primary products using zip-code primers.
3. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
RB1 microsatellites
p53 microsatellites
NM23 microsatellites
119 129 139 149 159 169 179 189 199 209 219
0
Allele 1
Allele 2
Allele 1
Allele 2
Allele 1
Allele 2
Loss of Heterozygosity

FIG. 22

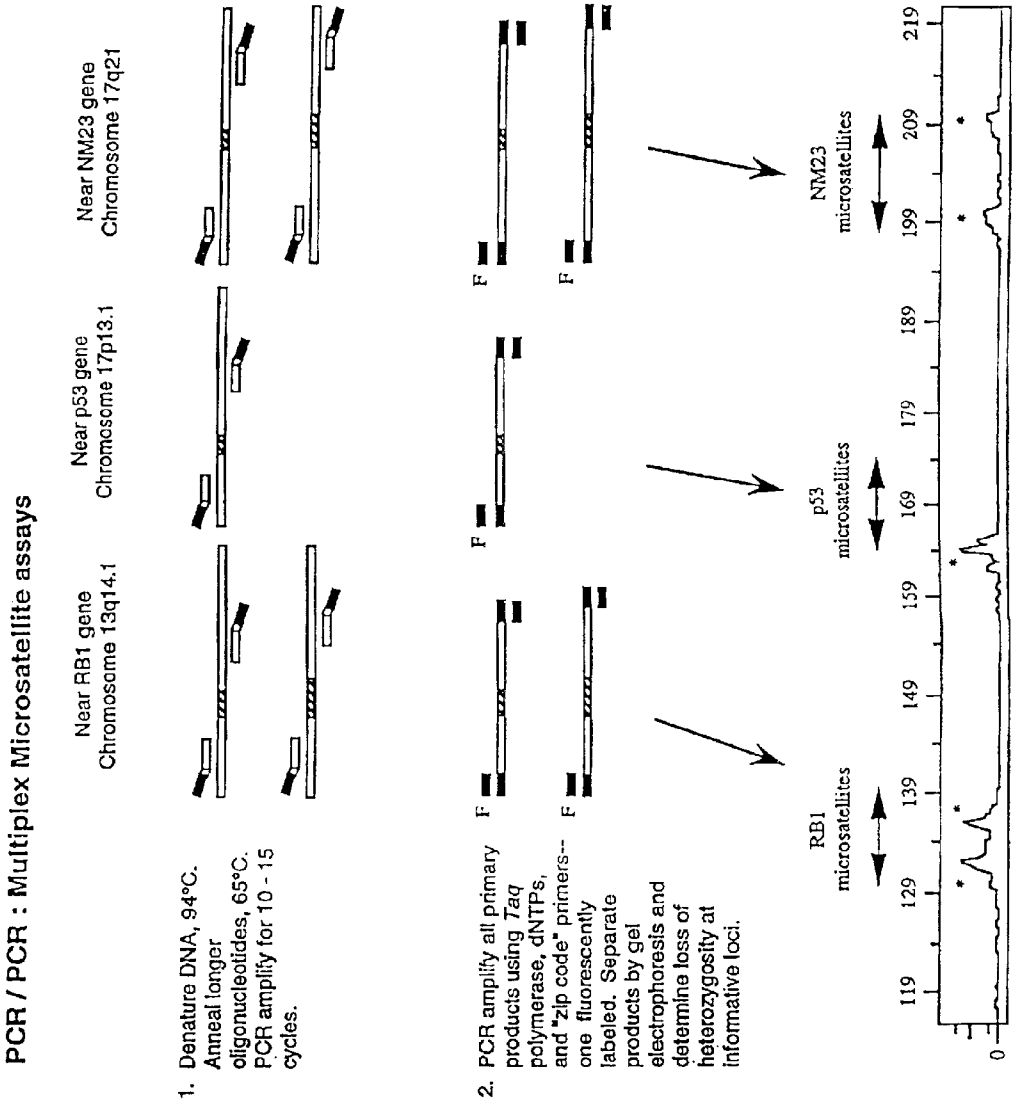

PCR / PCR : Multiplex Microsatellite assays
Near RB1 gene Chromosome 13q14.1
Near p53 gene Chromosome 17p13.1
Near NM23 gene Chromosome 17q21
1. Denature DNA, 94°C. Anneal longer oligonucleotides, 65°C. PCR amplify for 10 - 15 cycles.
2. PCR amplify all primary products using Taq polymerase, dNTPs, and "zip code" primers-- one fluorescently labeled. Separate products by gel electrophoresis and determine loss of heterozygosity at informative loci.
F
RB1 microsatellites
p53 microsatellites
NM23 microsatellites
119
129
139
149
159
169
179
189
199
209
219
0

FIG. 23

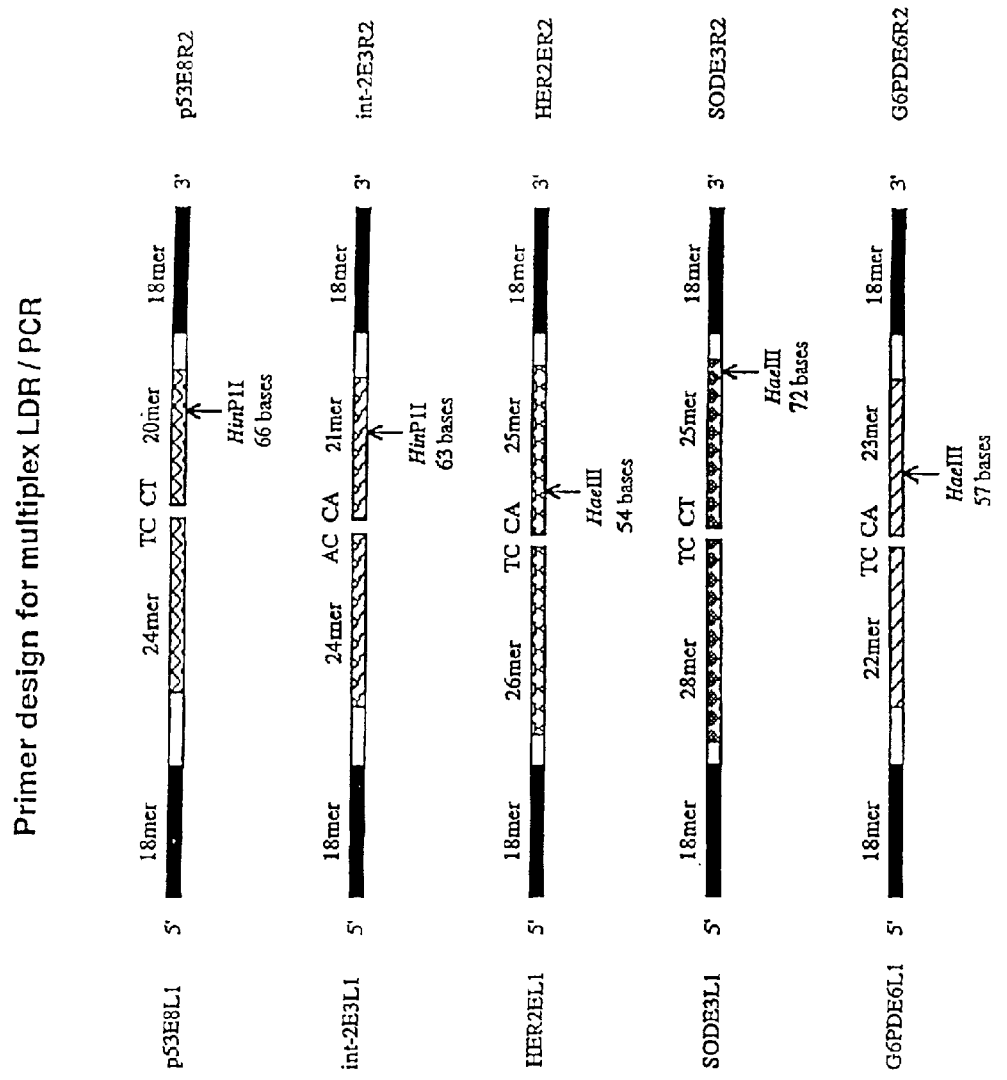

Primer design for multiplex LDR / PCR
p53E8L1
5'
18mer
24mer
TC CT
20mer
18mer
3'
p53E8R2
HinP1I
66 bases
int-2E3L1
5'
18mer
24mer
AC CA
21mer
18mer
3'
int-2E3R2
HinP1I
63 bases
HER2EL1
5'
18mer
26mer
TC CA
25mer
18mer
3'
HER2ER2
HaeIII
54 bases
SODE3L1
5'
18mer
28mer
TC CT
25mer
18mer
3'
SODE3R2
HaeIII
72 bases
G6PDE6L1
5'
18mer
22mer
TC CA
23mer
18mer
3'
G6PDE6R2
HaeIII
57 bases

FIG. 24

FIG. 25A

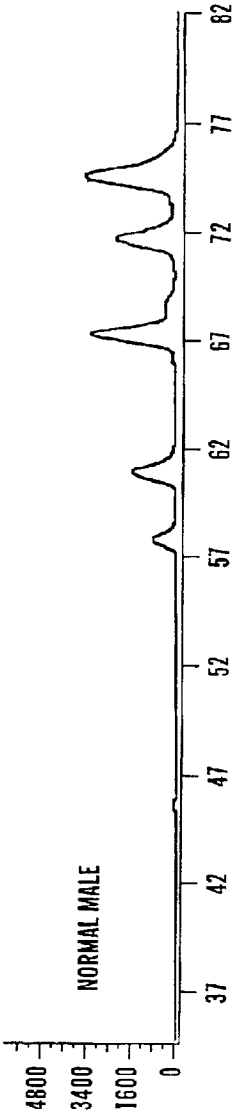


FIG. 25B

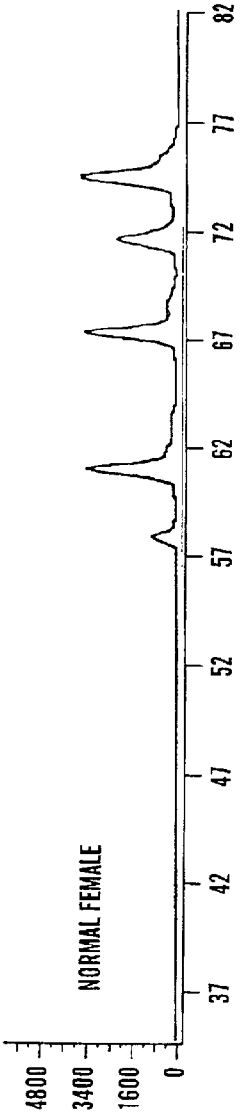


FIG. 25C

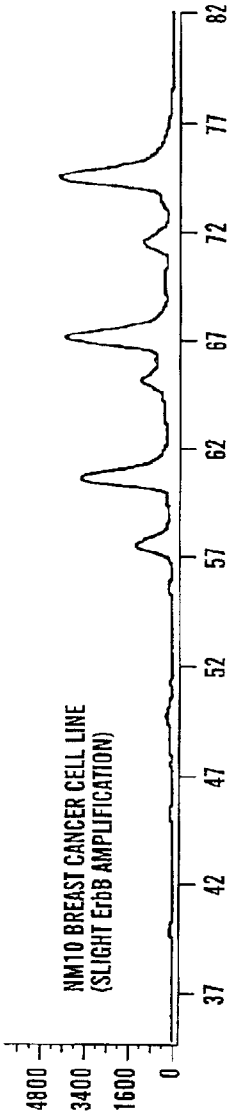


FIG. 25D

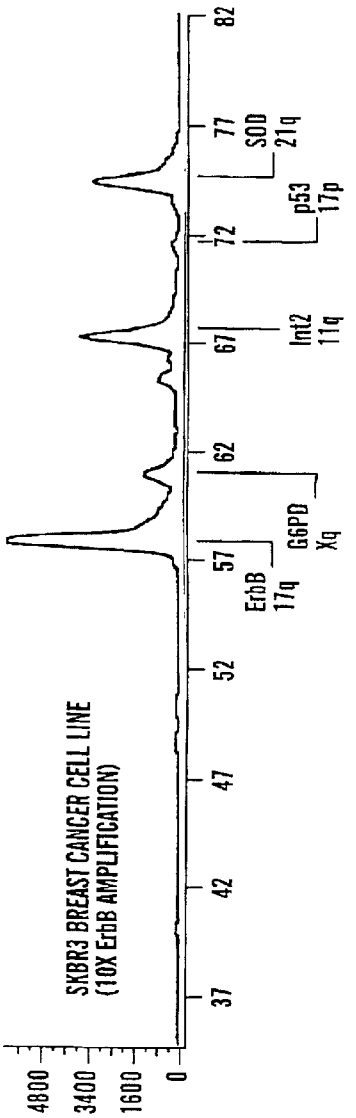


FIG. 26A

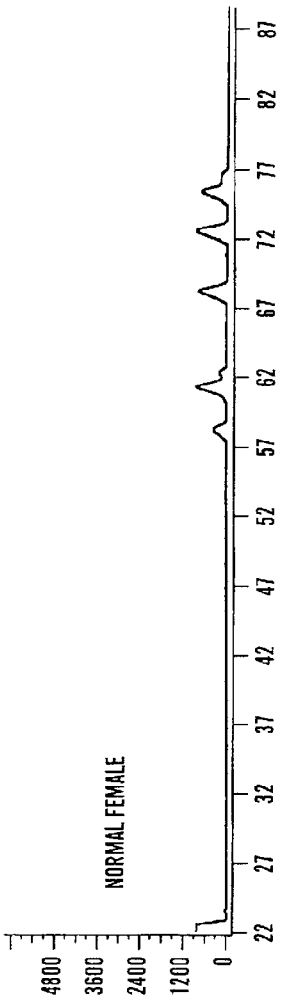


FIG. 26B

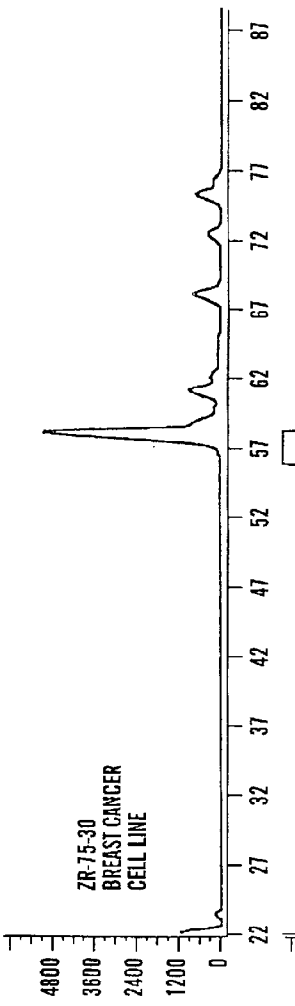


FIG. 26C

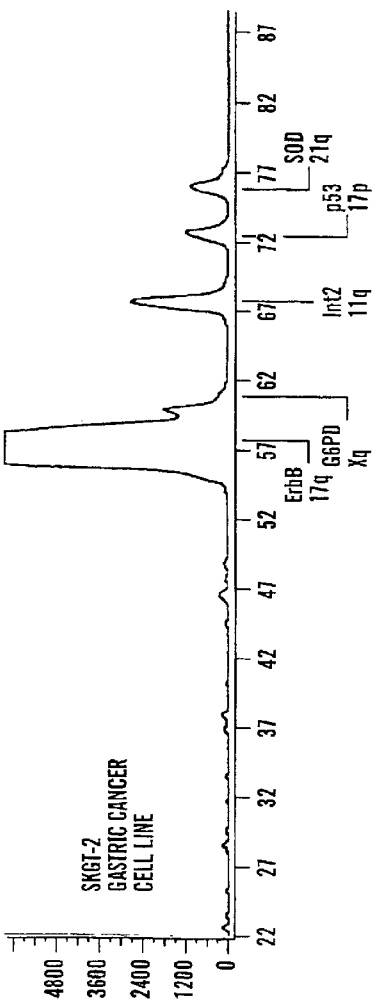


FIG. 27A

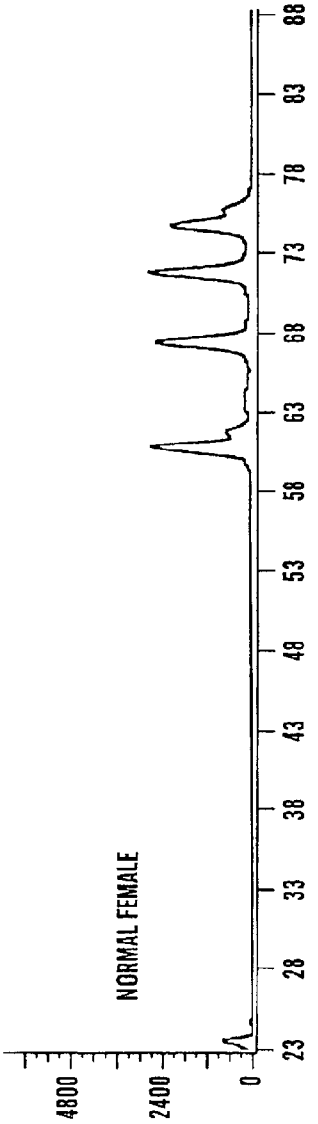


FIG. 27B

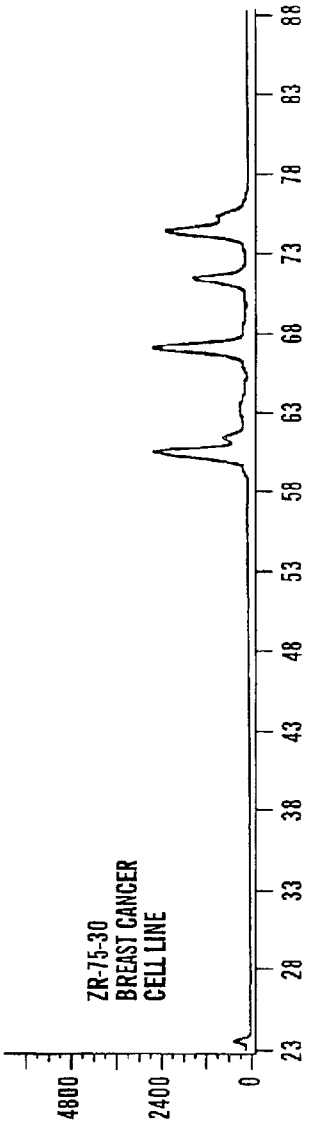
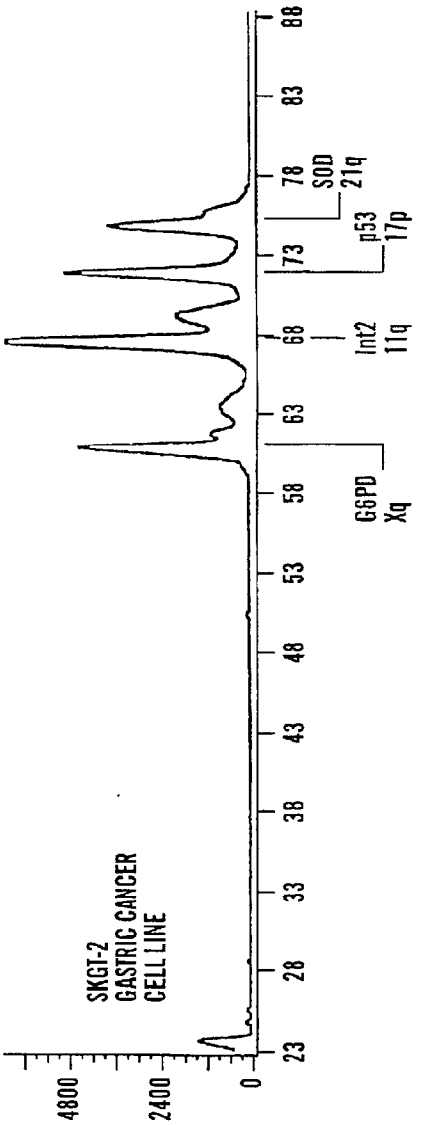


FIG. 27C



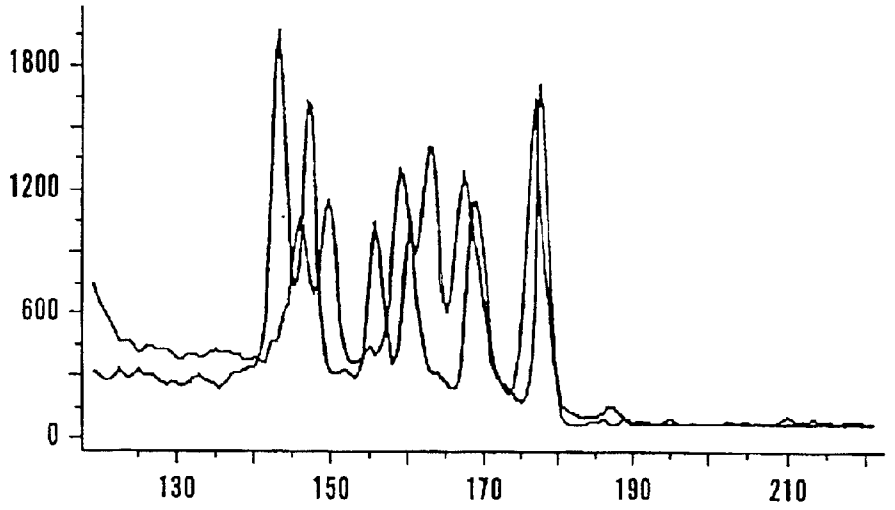


FIG. 28A

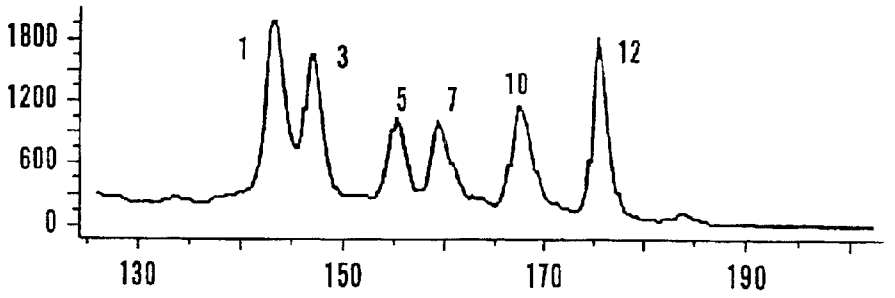


FIG. 28B

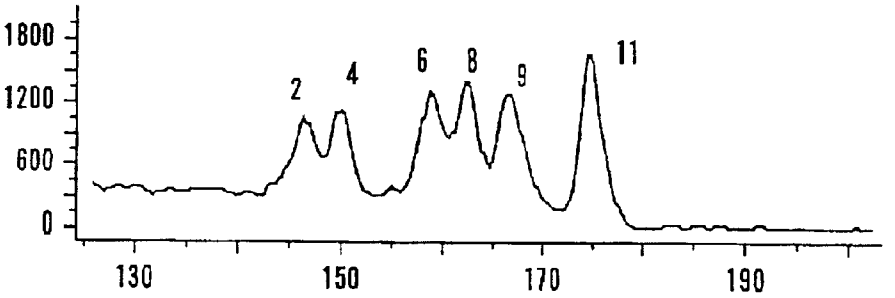


FIG. 28C

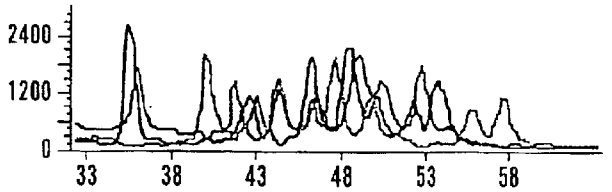


FIG. 29A

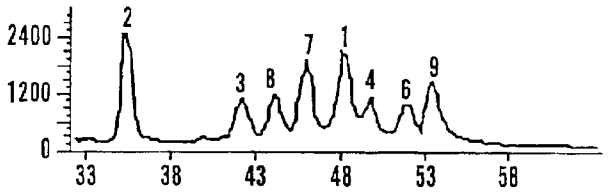


FIG. 29B

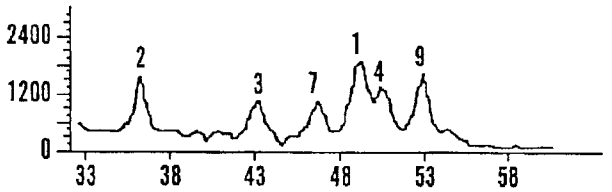


FIG. 29C

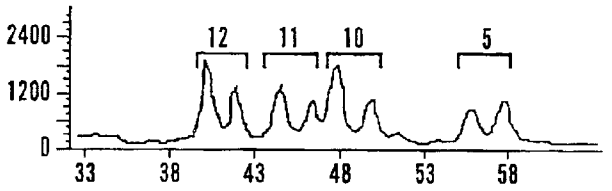


FIG. 29D

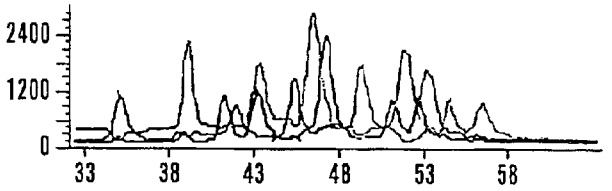


FIG. 29E

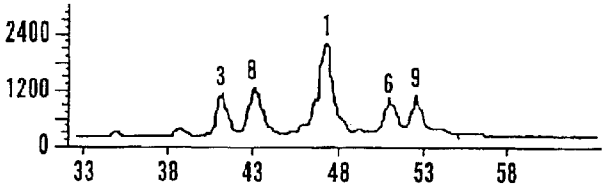


FIG. 29F

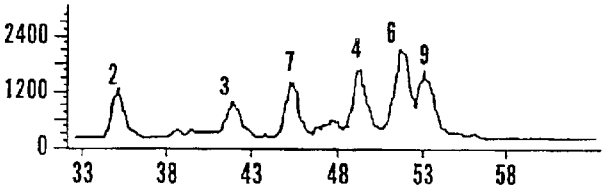


FIG. 29G

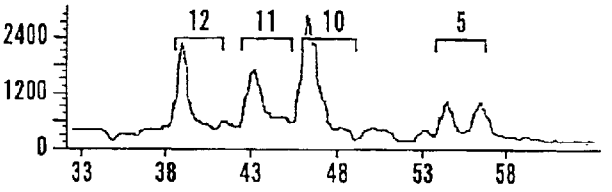


FIG. 29H

DETECTION OF NUCLEIC ACID SEQUENCE DIFFERENCES USING COUPLED LIGASE DETECTION AND POLYMERASE CHAIN REACTIONS

This application is a continuation of U.S. patent application Ser. No. 09/440,523, filed Nov. 15, 1999, U.S. Pat. No. 6,268,148, which is a divisional of U.S. patent application Ser. No. 08/864,473, filed May 28, 1997, now U.S. Pat. No. 6,027,889, and claims the benefit of U.S. Provisional Patent Application Serial No. 60/018,532, filed May 29, 1996.

This invention was developed with government funding under National Institutes of Health Grant No. GM41337-06. The U.S. Government may retain certain rights.

FIELD OF THE INVENTION

The present invention relates to the detection of nucleic acid sequence differences using coupled ligase detection reaction ("LDR") and polymerase chain reaction ("PCR"). One aspect of the present invention involves use of a ligase detection reaction coupled to a polymerase chain reaction. Another aspect of the present invention relates to the use of a primary polymerase chain reaction coupled to a secondary polymerase chain reaction coupled to a ligase detection reaction. A third aspect of the present invention involves a primary polymerase chain reaction coupled to a secondary polymerase chain reaction.

BACKGROUND OF THE INVENTION

Multiplex Detection

Large-scale multiplex analysis of highly polymorphic loci is needed for practical identification of individuals, e.g., for paternity testing and in forensic science (Reynolds et al., *Anal. Chem.,* 63:2–15 (1991)), for organ-transplant donor-recipient matching (Buyse et al., *Tissue Antigens,* 41:1–14 (1993) and Gyllensten et al., *PCR Meth. Appl,* 1:91–98 (1991)), for genetic disease diagnosis, prognosis, and prenatal counseling (Chamberlain et al., *Nucleic Acids Res.,* 16:11141–11156 (1988) and L. C. Tsui, *Human Mutat.,* 1:197–203 (1992)), and the study of oncogenic mutations (Hollstein et al., *Science,* 253:49–53 (1991)). In addition, the cost-effectiveness of infectious disease diagnosis by nucleic acid analysis varies directly with the multiplex scale in panel testing. Many of these applications depend on the discrimination of single-base differences at a multiplicity of sometimes closely spaced loci.

A variety of DNA hybridization techniques are available for detecting the presence of one or more selected polynucleotide sequences in a sample containing a large number of sequence regions. In a simple method, which relies on fragment capture and labeling, a fragment containing a selected sequence is captured by hybridization to an immobilized probe. The captured fragment can be labeled by hybridization to a second probe which contains a detectable reporter moiety.

Another widely used method is Southern blotting. In this method, a mixture of DNA fragments in a sample is fractionated by gel electrophoresis, then fixed on a nitrocellulose filter. By reacting the filter with one or more labeled probes under hybridization conditions, the presence of bands containing the probe sequences can be identified. The method is especially useful for identifying fragments in a restriction-enzyme DNA digest which contains a given probe sequence and for analyzing restriction-fragment length polymorphisms ("RFLPs").

Another approach to detecting the presence of a given sequence or sequences in a polynucleotide sample involves

selective amplification of the sequence(s) by polymerase chain reaction. U.S. Pat. No. 4,683,202 to Mullis, et al. and R. K. Saiki, et al., *Science* 230:1350 (1985). In this method, primers complementary to opposite end portions of the selected sequence(s) are used to promote, in conjunction with thermal cycling, successive rounds of primer-initiated replication. The amplified sequence(s) may be readily identified by a variety of techniques. This approach is particularly useful for detecting the presence of low-copy sequences in a polynucleotide-containing sample, e.g., for detecting pathogen sequences in a body-fluid sample.

More recently, methods of identifying known target sequences by probe ligation methods have been reported. U.S. Pat. No. 4,883,750 to N. M. Whiteley, et al., D. Y. Wu, et al., *Genomics* 4:560 (1989), U. Landegren, et al., *Science* 241:1077 (1988), and E. Winn-Deen, et al., *Clin. Chem.* 37:1522 (1991). In one approach, known as oligonucleotide ligation assay ("OLA"), two probes or probe elements which span a target region of interest are hybridized to the target region. Where the probe elements basepair with adjacent target bases, the confronting ends of the probe elements can be joined by ligation, e.g., by treatment with ligase. The ligated probe element is then assayed, evidencing the presence of the target sequence.

In a modification of this approach, the ligated probe elements act as a template for a pair of complementary probe elements. With continued cycles of denaturation, hybridization, and ligation in the presence of pairs of probe elements, the target sequence is amplified linearly, allowing very small amounts of target sequence to be detected and/or amplified. This approach is referred to as ligase detection reaction. When two complementary pairs of probe elements are utilized, the process is referred to as the ligase chain reaction which achieves exponential amplification of target sequences. F. Barany, "Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase," *Proc. Nat'l Acad. Sci. USA,* 88:189–93 (1991) and F. Barany, "The Ligase Chain Reaction (LCR) in a PCR World," *PCR Methods and Applications,* 1:5–16 (1991).

Another scheme for multiplex detection of nucleic acid sequence differences is disclosed in U.S. Pat. No. 5,470,705 to Grossman et. al. where sequence-specific probes, having a detectable label and a distinctive ratio of charge/translational frictional drag, can be hybridized to a target and ligated together. This technique was used in Grossman, et. al., "High-density Multiplex Detection of Nucleic Acid Sequences: Oligonucleotide Ligation Assay and Sequence-coded Separation," *Nucl. Acids Res.* 22(21):4527–34 (1994) for the large scale multiplex analysis of the cystic fibrosis transmembrane regulator gene.

Jou, et. al., "Deletion Detection in Dystrophia Gene by Multiplex Gap Ligase Chain Reaction and Immunochromatographic Strip Technology," *Human Mutation* 5:86–93 (1995) relates to the use of a so called "gap ligase chain reaction" process to amplify simultaneously selected regions of multiple exons with the amplified products being read on an immunochromatographic strip having antibodies specific to the different haptens on the probes for each exon.

There is a growing need (e.g., in the field of genetic screening) for methods useful in detecting the presence or absence of each of a large number of sequences in a target polynucleotide. For example, as many as 400 different mutations have been associated with cystic fibrosis. In screening for genetic predisposition to this disease, it is optimal to test all of the possible different gene sequence mutations in the subject's genomic DNA, in order to make a positive identification of "cystic fibrosis". It would be ideal

to test for the presence or absence of all of the possible mutation sites in a single assay. However, the prior-art methods described above are not readily adaptable for use in detecting multiple selected sequences in a convenient, automated single-assay format.

Solid-phase hybridization assays require multiple liquid-handling steps, and some incubation and wash temperatures must be carefully controlled to keep the stringency needed for single-nucleotide mismatch discrimination. Multiplexing of this approach has proven difficult as optimal hybridization conditions vary greatly among probe sequences.

Developing a multiplex PCR process that yields equivalent amounts of each PCR product can be difficult and laborious. This is due to variations in the annealing rates of the primers in the reaction as well as varying polymerase extension rates for each sequence at a given Mg^{2+} concentration. Typically, primer, Mg^{2+}, and salt concentrations, along with annealing temperatures are adjusted in an effort to balance primer annealing rates and polymerase extension rates in the reaction. Unfortunately, as each new primer set is added to the reaction, the number of potential amplicons and primer dimers which could form increase exponentially. Thus, with each added primer set, it becomes increasingly more difficult and time consuming to work out conditions that yield relatively equal amounts of each of the correct products.

Allele-specific PCR products generally have the same size, and an assay result is scored by the presence or absence of the product band(s) in the gel lane associated with each reaction tube. Gibbs et al., *Nucleic Acids Res.,* 17:2437–2448 (1989). This approach requires splitting the test sample among multiple reaction tubes with different primer combinations, multiplying assay cost. PCR has also discriminated alleles by attaching different fluorescent dyes to competing allelic primers in a single reaction tube (F. F. Chehab, et al., *Proc. Natl. Acad. Sci. USA,* 86:9178–9182 (1989)), but this route to multiplex analysis is limited in scale by the relatively few dyes which can be spectrally resolved in an economical manner with existing instrumentation and dye chemistry. The incorporation of bases modified with bulky side chains can be used to differentiate allelic PCR products by their electrophoretic mobility, but this method is limited by the successful incorporation of these modified bases by polymerase, and by the ability of electrophoresis to resolve relatively large PCR products which differ in size by only one of these groups. Livak et al., *Nucleic Acids Res.,* 20:4831–4837 (1989). Each PCR product is used to look for only a single mutation, making multiplexing difficult.

Ligation of allele-specific probes generally has used solid-phase capture (U. Landegren et al., *Science,* 241:1077–1080 (1988); Nickerson et al., *Proc. Natl. Acad. Sci. USA,* 87:8923–8927 (1990)) or size-dependent separation (D. Y. Wu, et al., *Genomics,* 4:560–569 (1989) and F. Barany, *Proc. Natl. Acad. Sci.,* 88:189–193 (1991)) to resolve the allelic signals, the latter method being limited in multiplex scale by the narrow size range of ligation probes. Further, in a multiplex format, the ligase detection reaction alone cannot make enough product to detect and quantify small amounts of target sequences. The gap ligase chain reaction process requires an additional step—polymerase extension. The use of probes with distinctive ratios of charge/translational frictional drag for a more complex multiplex will either require longer electrophoresis times or the use of an alternate form of detection.

The need thus remains for a rapid single assay format to detect the presence or absence of multiple selected sequences in a polynucleotide sample.

Microsatellite Analysis

Tandem repeat DNA sequences known as microsatellites represent a very common and highly polymorphic class of genetic elements within the human genome. These microsatellite markers containing small repeat sequences have been used for primary gene mapping and linkage analysis. Weber, J. L. et al., *Am. J. Hum. Genet.* 44:388–396 (1989); Weissenbach, J. et al., *Nature (London)* 359:794–800 (1992). PCR amplification of these repeats allows rapid assessment for loss of heterozygosity and can greatly simplify procedures for mapping tumor suppressor genes. Ruppert, J. M., et al., *Cancer Res.* 53:5093–94 (1993); van der Riet, et al., *Cancer Res.* 54:1156–58 (1994); Nawroz, H., et al., *Cancer Res.* 54: 1152–55 (1994); Cairns, P., et al., *Cancer Res.* 54:1422–24 (1994). More recently, they have been used to identify specific mutations in certain inherited disorders including Huntington disease, fragile X syndrome, myotonic dystrophy, spinocerebellar ataxia type I, spinobulbar muscular atrophy, and hereditary dentatorubral-pallidoluysian atrophy. The Huntington's Disease Collaborative Research Group *Cell* 72:971–83 (1993); Kremer, E. J., et al., *Science* 252:1711–14 (1991); Imbert, G., et al., *Nat. Genet.* 4:72–76 (1993); Orr, H. T., et al., *Nat. Genet.* 4:221–226 (1993); Biancalana, V., et al., *Hum. Mol. Genet.* 1:255–258 (1992); Chung, M.-Y., et al., *Nat. Genet.* 5:254–258 (1993); Koide, R., et al., *Nat. Genet.* 6:9–13 (1994). These inherited disorders appear to arise from the expansion of trinucleotide repeat units within susceptible genes. A more widespread microsatellite instability, demonstrated by expansion or deletion of repeat elements in neoplastic tissues, was first reported in colorectal tumors. Peinado, M. A., et al. *Proc. Natl. Acad. Sci. USA* 89:10065–69 (1992); Ionov, Y., *Nature* (*London*) 363:558–61 (1993); Thibodeau, S. N., et al., *Science* 260: 816–819 (1993) and later in several other tumor types (Risinger, J. I., *Cancer Res.* 53:5100–03 (1993); Han, H.-J., et al., *Cancer Res.* 53:5087–89 (1993); Peltomäki, P., *Cancer Res.* 53:5853–55 (1993); Gonzalez-Zulueta, M., et al., *Cancer Res.* 53:5620–23 (1993); Merlo, A., et al., *Cancer Res.* 54:2098–2101 (1994)). In hereditary nonpolyposis colorectal carcinoma patients, this genetic instability is apparently due to inherited and somatic mutations in mismatch repair genes. Leach, F., et al., *Cell* 75:1215–1225 (1993); Fishel, R., et al., *Cell* 75: 1027–38 (1993); Papadopoulos, N., et al., *Science* 263:1625–29 (1994); Bronner, C. E., et al., *Nature* (*London*) 368:258–61 (1994).

PCR is commonly used for microsatellite analysis in identifying both the appearance of new polymorphisms and the loss of heterozygosity in cancer detection. L. Mao, et. al., "Microsatellite Alterations as Clonal Markers for the Detection of Human Cancer," *Proc. Nat'l Acad. Sci USA* 91(21): 9871–75 (1994); L. Mao, et. al., "Molecular Detection of Primary Bladder Cancer by Microsatellite Analysis," *Science* 271:659–62 (1996); D. Radford, et. al., "Allelotyping of Ductal Carcinoma in situ of the Breast: Detection of Loci on 8p, 13q, 161, 17p and 17q," *Cancer Res.* 55(15): 3399–05 (1995). In using PCR for such purposes, each PCR reaction is run individually and separated on a sequencing gel.

Although these references demonstrate that PCR has application to diagnosis and prognosis of certain cancers, this type of analysis is deficient, because it does not permit a high throughput and requires size separation. In addition, there are problems with PCR slippage, causing researchers to shift to tri-, tetra-, and higher nucleotide repeat units, making cancer detection more difficult.

Microsatellite markers have also been used for colon cancer detection (L. Cawkwell, et. al., "Frequency of Allele

Loss of DCC, p53, RB1, WT1, NF1, NM23, and APC/MCC in Colorectal Cancer Assayed by Fluorescent Multiplex Polymerase Chain Reaction," *Br. J. Cancer* 70(5): 813–18 (1994)) and for genome mapping (P. Reed, et. al., "Chromosome-specific Microsatellite Sets for Fluorescent-Based, Semi-Automated Genome Mapping," *Nat. Genet.* 7(3): 390–95 (1994)). However, the key to such multiplex processes is the ability to perform them in a single reaction tube. Conventional multiplex microsatellite marker approaches require careful attention to primer concentrations and amplification conditions. Although PCR products can be pooled in sets, this requires a prerun on agarose gels to insure that the mixture has about equal amounts of DNA in each band.

Human Identification

PCR has also been used for human identification, such as paternity testing, criminal investigations, and military personnel identification. A. Syvanen et. al., "Identification of Individuals by Analysis of Biallelic DNA Markers, Using PCR and Solid-Phase Mini-Sequencing" *Am. J. Hum. Genet.* 52(1): 46–59 (1993) describes a mini-sequencing approach to human identification. The technique requires PCR amplification of individual markers with at most 4 PCR reactions being carried out at a time in a single PCR tube. Mini-sequencing is carried out to determine individual polymorphisms.

Coupled Processes

G. Deng, et. al., "An Improved Method of Competitive PCR for Quantitation of Gene Copy Number," *Nucl. Acids Res.* 21:4848–49 (1993) describes a competitive PCR process. Here, two PCR steps are utilized with different sets of primers being used for each gene and its equivalent standard.

T. Msuih, et. al., "Novel, Ligation-Dependent PCR Assay for Detection of Hepatitis C. Virus in Serum," *J. Clin Microbio.* 34:501–07 (1996) and Y. Park, et. al., "Detection of HCV RNA Using Ligation-Dependent Polymerase Chain Reaction in Formalin-Fixed Paraffin-Embedded Liver Tissue" (submitted) describe the use of a LDR/PCR process in work with RNA.

SUMMARY OF THE INVENTION

The present invention is directed to the detection of nucleic acid sequence differences using coupled LDR and PCR processes. The present invention can be carried out in one of the following 3 embodiments: (1) LDR coupled to PCR; (2) primary PCR coupled to secondary PCR coupled to LDR; and (3) primary PCR coupled to secondary PCR. Each of these embodiments have particular applicability in detecting certain characteristics. However, each requires the use of coupled reactions for multiplex detection of nucleic acid sequence differences where oligonucleotides from an early phase of each process contain sequences which may be used by oligonucleotides from a later phase of the process.

I. Primary PCR/Secondary PCR/LDR Process

One aspect of the present invention relates to a method for identifying two or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. This method involves a first polymerase chain reaction phase, a second polymerase chain reaction phase, and a ligase detection reaction phase. This process involves analyzing a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences.

In the first polymerase chain reaction phase, one or more primary oligonucleotide primer groups are provided. Each group comprises one or more primary oligonucleotide primer sets with each set having a first nucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion, and a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion. The first oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion and the second oligonucleotide primers of each set in the same group contain the same 5' upstream primer-specific portion. The oligonucleotide primers in a particular set are suitable for hybridization on complementary strands of a corresponding target nucleotide sequence to permit formation of a polymerase chain reaction product. However, there is a mismatch which interferes with formation of such a polymerase chain reaction product when the primary oligonucleotide primers hybridize to any other nucleotide sequence in the sample. The polymerase chain reaction products in a particular set may be distinguished from other polymerase chain reaction products in the same group or groups. The primary oligonucleotide primers, the sample, and the polymerase are blended to form a primary polymerase chain reaction mixture.

The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as substantially described above. During hybridization, target-specific portions of the primary oligonucleotide primers hybridize to the target nucleotide sequences. The extension treatment causes hybridized primary oligonucleotide primers to be extended to form primary extension products complementary to the target nucleotide sequence to which the primary oligonucleotide primers are hybridized.

Although the upstream secondary primer-specific portions of a primary oligonucleotide primer set are not present on the target DNA, their sequences are copied by the second and subsequent cycles of the primary polymerase chain reaction phase. As a result, the primary extension products produced after the second cycle have the secondary primer-specific portions on their 5' ends and the complement of primer-specific portion on their 3' ends.

Next, there is a second polymerase chain reaction phase. This phase involves providing one or a plurality of secondary oligonucleotide primer sets. Each set has a first secondary oligonucleotide primer containing the same sequence as the 5' upstream portion of the first primary oligonucleotide primer, and a second secondary oligonucleotide primer containing the same sequence as the 5' upstream portion of the second primary oligonucleotide primer from the same primary oligonucleotide primer set as the first primary oligonucleotide complementary to the first secondary primer. A set of secondary oligonucleotide primers may be used to amplify all of the primary extension products in a given group. The secondary oligonucleotide primers are blended with the primary extension products and the polymerase to form a secondary polymerase chain reaction mixture.

The secondary polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles having a denaturation treatment, a hybridization treatment, and an extension treatment, as substantially set forth above. During the hybridization treatment, the secondary oligonucleotide primers hybridize to the complementary sequences present on the primary extension products but not to the original target sequence. The extension treatment causes the hybridized secondary oligonucleotide primers to be extended to form secondary extension products complementary to the primary extension products.

The last phase of this aspect of the present invention involves a ligase detection reaction process. Here, a plurality of oligonucleotide probe sets are provided where each set has a first oligonucleotide probe, having a secondary extension product-specific portion and a detectable reporter label, and a second oligonucleotide probe, having a secondary extension product-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a complementary secondary extension product-specific portion. However, there is a mismatch which interferes with such ligation when the oligonucleotide probes are hybridized to any other nucleotide sequence present in the sample. The ligation product of oligonucleotide probes in a particular set may be distinguished from either probe or other ligation products. The plurality of oligonucleotide probe sets, the secondary extension products, and a ligase are blended to form a ligase detection reaction mixture.

The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles having a denaturation treatment and hybridization treatment substantially as described above. In the hybridization treatment, the oligonucleotide probe sets hybridize at adjacent positions in a base-specific manner to the respective secondary extension products if present. As a result, adjacent probes ligate to one another to form a ligation product sequence containing the detectable reporter label and the secondary extension product-specific portions connected together. The oligonucleotide probe sets may hybridize to nucleotide sequences other than their respective complementary secondary extension products but do not ligate together due to the presence of one or more mismatches and individually separate during the denaturation treatment. Following the ligase detection reaction cycles, the reporter labels of the ligation product sequences are detected which indicates the presence of one or more target nucleotide sequences in the sample.

The primary PCR/secondary PCR/LDR process of the present invention provides significant advantages over the use of PCR alone in the multiplex detection of single nucleotide and tandem repeat polymorphisms.

As noted above, the use of PCR alone requires heavy optimization of operating conditions in order to conduct multiplex detection procedures. Moreover, the quantity of oligonucleotide primers must be increased to detect greater numbers of target nucleotide sequences. However, as this occurs, the probability of target independent reactions (e.g., the primer-dimer effect) increases. In addition, the mutations must be known, false positives may be generated by polymerase extension off of normal template, closely-clustered sites due to interference of overlapping primers cannot undergo multiplex detection, single base or small insertions and deletions in small repeat sequences cannot be detected, and quantification of mutant DNA in high background of normal DNA is difficult. As a result, the number of target nucleotide sequences detected in a single multiplex PCR process is limited.

Direct sequencing requires enrichment of mutant samples in order to correct sequences, requires multiple reactions for large genes containing many exons, requires electrophoretic separation of products, is time consuming, and cannot be used to detect mutant DNA in less than 5% of background of normal DNA. When mini-sequencing, the mutation must be known, closely-clustered sites due to interference of overlapping primers cannot undergo multiplex detection, single base or small insertions and deletions in small repeat sequences cannot be detected, and four separate reactions are required. For allele-specific oligonucleotide hybridization ("ASO"), the mutation must be known, hybridization and washing conditions must be known, cross-reactivity is difficult to prevent, closely-clustered sites due to interference of overlapping primers cannot undergo multiplex detection, and mutant DNA cannot be detected in less than 5% of background of normal DNA. Primer-mediated RFLP requires electrophoretic separation to distinguish mutant from normal DNA, is of limited applicability to sites that may be converted into a restriction site, requires additional analysis to determine the nature of the mutation, and is difficult to use where the mutant DNA is in a high background of normal DNA. Single strand conformational polymorphism analysis ("SSCP") requires electrophoretic separation to distinguish mutant conformer from normal conformer, misses 30% of possible mutations, requires additional analysis to determine the nature of the mutation, and cannot distinguish mutations from silent polymorphisms. With dideoxynucleotide finger printing ("ddF"), it is difficult to detect mutations in a high background of normal DNA, electrophoretic separation is required to distinguish mutant conformer from normal conformer, additional analysis must be used to determine the nature of the mutation, and mutations cannot be distinguished from silent polymorphisms. Denaturing gradient gel electrophoresis ("DGGE") must electrophoretically separate mutant conformer from normal conformer, misses 30% of possible mutations, requires additional analysis to determine the nature of the mutation, cannot distinguish mutations from silent polymorphisms, and imposes technical challenges to reproducing previously achieved results. RNase mismatch cleavage requires additional analysis to determine the nature of the mutation, requires analysis of both strands to exclude RNase-resistant mismatches, and imposes difficulty in detecting mutations in a high background of normal DNA. Chemical mismatch cleavage cannot detect mutant DNA in less than 5% of background of normal DNA, and requires an analysis of both strands to detect all mutations. For T4 Endo VII mismatch cleavage, additional analysis is needed to determine the nature of the mutation, mutations cannot be distinguished from silent polymorphisms, endonuclease cleaves control DNA which necessitates careful interpretation of results, and it is difficult to detect mutations in a high background of normal DNA.

These problems are avoided in the primary PCR/secondary PCR/LDR process of the present invention which combines the sensitivity of PCR with the specificity of LDR. The primary PCR phase produces primary extension products with a secondary primer-specific portion. This initial phase is carried out under conditions effective to maximize production of primary extension products without obtaining the adverse effects sometimes achieved in a PCR-only process. In particular, the primary PCR phase of the present invention is carried out with 15 to 20 PCR cycles and utilizes less primer than would be utilized in a PCR-only process. The primary PCR phase of the present invention produces extension products in a varied and unpredictable way, because some target nucleotide sequences will be amplified well, while others will not. However, in the secondary PCR phase, all of the primary extension products are amplified approximately equally, because they all have the same secondary primer-specific portions. Target nucleotide sequences originally present in the sample will not be amplified by the secondary PCR phase, because such sequences do not contain a secondary primer-specific portion. As a result, the primary PCR/secondary PCR/LDR process of the present invention is able to achieve multiplex detection of hundreds of nucleotide sequence differences in

a single tube without undue customization of operating conditions for each particular sample being analyzed. Since the selection of mutant sequences is mediated by LDR rather than PCR, the primary PCR/secondary PCR/LDR process is less susceptible to false-positive signal generation. In addition, the primary PCR/secondary PCR/LDR process allows detection of closely-clustered mutations, detection of single base or small insertions and deletions in small repeat sequences, quantitative detection of less than 1% mutations in high background of normal DNA, and detection of ligation product sequences using addressable arrays. The only significant requirements are that the mutations be known and that a multitude of oligonucleotides be synthesized.

The ability to detect single nucleotide and tandem repeat polymorphisms is particularly important for forensic DNA identification and diagnosis of genetic diseases.

II. LDR/PCR Process

A second aspect of the present invention relates to a method for identifying one or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. This method has a ligase detection reaction phase followed by a polymerase chain reaction phase. This method involves providing a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences.

In the ligase detection reaction phase, one or more oligonucleotide probe sets are provided. Each set has a first oligonucleotide probe, having a target-specific portion and a 5' upstream primer-specific portion, and a second oligonucleotide probe, having a target-specific portion and a 3' downstream primer-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target nucleotide sequence. However, there is a mismatch which interferes with such ligation when they are hybridized to any other nucleotide sequence present in the sample. The sample, the plurality of oligonucleotide probe sets, and a ligase are blended together to form a ligase detection reaction mixture.

The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles. These cycles include a denaturation treatment and a hybridization treatment. In the denaturation treatment, any hybridized oligonucleotides are separated from the target nucleotide sequences. The hybridization treatment causes the oligonucleotide probe sets to hybridize at adjacent positions in a base-specific manner to their respective target nucleotide sequences if present in the sample. Once hybridized, the oligonucleotide probe sets ligate to one another to form a ligation product sequence. This product contains the 5' upstream primer-specific portion, the target-specific portions connected together, and the 3' downstream primer-specific portion. The ligation product sequence for each set is distinguishable from other nucleic acids in the ligase detection reaction mixture. The oligonucleotide probe sets hybridized to nucleotide sequences in the sample other than their respective target nucleotide sequences but do not ligate together due to a presence of one or more mismatches and individually separate during the subsequent denaturation treatment.

In the polymerase chain reaction, one or a plurality of oligonucleotide primer sets are provided. Each set has an upstream primer containing the same sequence as the 5' upstream primer-specific portion of the ligation product sequence and a downstream primer complementary to the 3' downstream primer-specific portion of the ligation product sequence, where one primer has a detectable reporter label. The ligase detection reaction mixture is blended with the one or a plurality of oligonucleotide primer sets and the polymerase to form a polymerase chain reaction mixture.

The polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles which include a denaturation treatment, a hybridization treatment, and an extension treatment. During the denaturation treatment, hybridized nucleic acid sequences are separated. The hybridization treatment causes primers to hybridize to their complementary primer-specific portions of the ligation product sequence. During the extension treatment, hybridized primers are extended to form extension products complementary to the sequences to which the primers are hybridized. In a first cycle of the polymerase chain reaction phase, the downstream primer hybridizes to the 3' downstream primer-specific portion of the ligation product sequence and is extended to form an extension product complementary to the ligation product sequence. In subsequent cycles the upstream primer hybridizes to the 5' upstream primer-specific portion of the extension product complementary to the ligation product sequence and the downstream primer hybridizes to the 3' downstream portion of the ligation product sequence.

Following the polymerase chain reaction phase of this process, the reporter labels are detected and the extension products are distinguished to indicate the presence of one or more target nucleotide sequences in the sample.

One embodiment of the LDR/PCR process of the present invention achieves improved results over the use of LDR alone in measuring the number of gene copies in a cell (i.e. gene dosage). When LDR alone is utilized, it is difficult to produce sufficient target copies which are needed ultimately to quantify a plurality of genes.

In another embodiment of the LDR/PCR process of the present invention, the LDR phase ligation product sequences are produced in a ratio proportional to the ratio of the genes from which they were derived within the sample. By incorporation of the same primer-specific portions in the oligonucleotide probes for the LDR phase, the PCR phase amplifies ligation product sequences to the same degree so that their proportionality is maintained. Target sequences originally found in the sample being analyzed are not amplified by the PCR phase, because such target sequences do not contain PCR primer-specific portions. In addition, since only the oligonucleotide primers for the PCR phase have reporter labels, only extension products with those labels will be detected.

Determination of variation in gene dosage is important in a number of biomedical applications.

Males differ in gene dosage from females for those genes located on the X chromosome, women having two copies while men have one copy. Women may be carriers of deletions along the X chromosome. If the deleted region of the X chromosome included one or more genes, then the woman has only one copy of these genes in the corresponding, non-deleted area of the other X chromosome. The result (having one copy of an X-linked gene) is similar to the situation in male cells and is usually tolerated without manifestations of an inherited disorder. However, if the woman's son inherits her deleted X chromosome, he will have no copies of the genes in the deletion region and suffer from the X-linked disorder related to the absence of the gene. The detection of chromosomal deletions, therefore, is one application of the LDR/PCR process of the present invention.

Congenital chromosomal disorders occur when a fertilized egg has an abnormal compliment of chromosomes. The most common congenital chromosomal disorder is Down Syndrome, which occurs when there is an additional chromosome 21 in each cell, designated 47,XX+21 or 47, XY+21. The LDR/PCR process of the present invention can be designed to identify congenital chromosomal disorders.

The LDR/PCR process of the present invention is also useful for distinguishing polymorphisms in mononucleotide and di-nucleotide repeat sequences. It will also be useful in distinguishing minor populations of cells containing unique polymorphisms for detection of clonality.

The LDR/PCR process can be used with both gel and non-gel (i.e. DNA array) technologies. It allows multiplex analysis of many gene amplifications and deletions simultaneously, allows quantitative analysis, and does not require an external standard. The only relatively minor challenge presented by the LDR/PCR process is that it is difficult to use in determining the boundaries of large deletions in chromosomes.

By contrast, microsatellite marker analysis cannot be used to detect small regions that are deleted or amplified, is not compatible with simultaneous detection of amplified regions, and depends on the availability of informative markers. Competitive PCR (i.e. differential PCR) cannot be used in a multiplex format to detect several deletions and amplifications simultaneously, and is not particularly accurate. Southern hybridization is time consuming, labor intensive, is not amenable to multiplexing due to the need for multiple steps for each probe tested, and requires large quantities of DNA. Fluorescent in situ hybridization ("FISH") requires specialized expertise, is time consuming, and requires large probes to analyze for suspected deleted or amplified regions. Thus, the LDR/PCR-process of the present invention constitutes a significant advance over prior processes.

III. Primary PCR/Secondary PCR Process

A third aspect of the present invention also involves a method for identifying two or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in one or more target nucleotide sequences. This method involves subjecting a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences to two successive polymerase chain reaction phases.

For the first polymerase chain reaction phase, one or more primary oligonucleotide primer groups are provided where each group comprises two or more primary oligonucleotide primer sets. Each set has a first oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primary-specific portion, and a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion. The first oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion and the second oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion. The oligonucleotide primers in a particular set are suitable for hybridization on complementary strands of a corresponding target nucleotide sequence to permit formation of a polymerase chain reaction product. However, there is a mismatch which interferes with formation of such a polymerase chain reaction product when the primary oligonucleotide primers hybridize to any other nucleotide sequence present in the sample. The polymerase chain reaction products in a particular set may be distinguished from other polymerase chain reaction products with the same group or other groups. The primary oligonucleotide primers are blended with the sample and the polymerase to form a primary polymerase chain reaction mixture.

The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as described above. During the hybridization treatment, the target-specific portion of a primary oligonucleotide primer is hybridized to the target nucleotide sequences. In the extension treatment, the hybridized primary oligonucleotide primers are extended to form primary extension products complementary to the target nucleotide sequence to which the primary oligonucleotide primer is hybridized.

Although the upstream secondary primer-specific portions of a primary oligonucleotide primer set are not present on the target DNA, their sequences are copied by the second and subsequent cycles of the primary polymerase chain reaction phase. As a result, the primary extension products produced after the second and subsequent cycles have the secondary primer-specific portions on their 5' ends and the complement of primer-specific portion on their 3' ends.

In the second polymerase chain reaction phase of this aspect of the present invention, one or a plurality of secondary oligonucleotide primer sets are provided. Each set has a first secondary primer having a detectable reporter label and containing the same sequence as the 5' upstream portion of a first primary oligonucleotide primer, and a second secondary primer containing the same sequence as the 5' upstream primer of the second primary oligonucleotide primer from the same primary oligonucleotide primer set as the first primary oligonucleotide complementary to the first secondary primer. A set of secondary oligonucleotide primers amplify the primary extension products in a given group. The secondary oligonucleotide primers are blended with the primary extension products and the polymerase to form a secondary polymerase chain reaction mixture.

The secondary polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as described above. In the hybridization treatment, the secondary oligonucleotide primers are hybridized to the primary extension products, while the extension treatment causes the hybridized secondary oligonucleotide primers to be extended to form secondary extension products complementary to the primary extension products. After subjecting the secondary polymerase chain reaction mixture to the two or more polymerase chain reaction cycles, the labelled secondary extension products are detected. This indicates the presence of one or more target nucleotide sequences in the sample.

The primary PCR/secondary PCR process of the present invention provides significant advantages over Southern hybridization, competitive PCR, and microsatellite marker analysis in detecting nucleotide deletions which cause a loss of heterozygosity. Although Southern hybridization is more accurate than competitive PCR, it is quite labor intensive, requires large amounts of DNA, and neither technique can be multiplexed. Current multiplex microsatellite marker approaches require careful attention to primer concentrations and amplification conditions.

The primary PCR/secondary PCR process of the present invention overcomes these difficulties encountered in the prior art. In the primary PCR phase, the primary oligonucleotide primers flank dinucleotide or other repeat sequences and include a secondary primer-specific portion. The primary PCR phase is carried out at low concentrations of these

primers to allow several loci to undergo amplification at the same time. The secondary PCR phase causes amplification to continue at the same rate with target-specific secondary primers being selected to space one set of microsatellite markers from the adjacent set. The primary PCR/secondary PCR process can be used to carry out multiplex detection in a single PCR tube and with single gel lane analysis.

This aspect of the present invention is useful in carrying out a microsatellite marker analysis to identify nucleotide deletions in a gene. Such multiplex detection can be carried out in a single reaction tube. However, the primary PCR/secondary PCR process cannot distinguish amplifications from deletions, so, when making such distinctions, this process must be used in conjunction with the above-described LDR/PCR process or a differential PCR process.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a flow diagram depicting a primary PCR/secondary PCR/LDR process for detection of germline mutations, such as point mutations, by electrophoresis or capture on an addressable array. Note that the term "zip-code" which appears in FIG. **1** and other drawings refers to a sequence specific to a subsequently used primer or probe but not to either the target sequence or other genome sequences.

FIG. **2** is a flow diagram depicting a primary PCR/secondary PCR/LDR process for detection of biallelic polymorphisms by electrophoresis or capture on an addressable array.

FIG. **3** is a flow diagram depicting a primary PCR/secondary PCR/LDR process for detection of cancer-associated mutations by electrophoresis or capture on an addressable array.

FIG. **4** is a schematic diagram depicting a primary PCR/secondary PCR/LDR process for detection of biallelic polymorphisms.

FIG. **5** is a schematic diagram depicting a primary PCR/secondary PCR/LDR process for detection of allelic differences using LDR oligonucleotide probes which distinguish all possible bases at a given site.

FIG. **6** is a schematic diagram depicting a primary PCR/secondary PCR/LDR process for detection of the presence of any possible base at two nearby sites using LDR oligonucleotide probes which distinguish all possible bases at a given site.

FIG. **7** is a schematic diagram depicting a primary PCR/secondary PCR/LDR process for detection of cancer-associated mutations at adjacent alleles.

FIG. **8** is a flow diagram depicting an LDR/PCR process with and without restriction endonuclease digestion using electrophoresis detection.

FIG. **9** is a flow diagram depicting an LDR/PCR process using detection on an addressable array using gene-specific addresses.

FIG. **10** is a schematic diagram depicting an LDR/PCR process for multiplex detection of gene amplifications and deletions.

FIG. **11** is a schematic diagram depicting an allele specific problem for an LDR/PCR process.

FIG. **12** is a schematic diagram depicting a solution for the allele specific problem for an LDR/PCR process which is shown in FIG. **11**.

FIG. **13** is a flow diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of biallelic polymorphisms by electrophoresis or capture on an addressable array.

FIG. **14** is a flow diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of cancer-associated mutations by electrophoresis or capture on an addressable array.

FIG. **15** is a schematic diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of allele specific mutations and polymorphisms.

FIG. **16** is a schematic diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of mononucleotide repeat polymorphisms.

FIG. **17** is a schematic diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of mononucleotide repeat polymorphisms which are in low abundance.

FIG. **18** is a flow diagram depicting the detection of polymorphisms using an LDR/PCR process with an intermediate sequenase extension phase and a uracil N-glycosylase digestion phase after the LDR phase and before the PCR phase and with detection by electrophoresis or an addressable array.

FIG. **19** is a flow diagram depicting the detection of cancer using an LDR/PCR process with an intermediate sequenase extension phase and a uracil N-glycosylase digestion phase after the LDR phase and before the PCR phase and with detection by electrophoresis or an addressable array.

FIG. **20** is a schematic diagram depicting detection of mononucleotide repeats using an LDR/PCR process with an intermediate sequenase amplification phase and a uracil N-glycosylase digestion phase after the LDR phase and before the PCR phase.

FIG. **21** is a schematic diagram depicting detection of mononucleotide repeat polymorphisms which are in low abundance using an LDR/PCR process with an intermediate sequenase amplification phase and a uracil N-glycosylase digestion phase after the LDR phase and before the PCR phase.

FIG. **22** is a flow diagram depicting a primary PCR/secondary PCR process for detection of microsatellite repeats.

FIG. **23** is a schematic diagram depicting a primary PCR/secondary PCR process for multiplex detection of insertions and deletions in microsatellite repeats.

FIG. **24** shows the design of LDR oligonucleotide probes for quantification of gene amplifications and deletions in an LDR/PCR process.

FIGS. **25**A–D show electropherogram results for an LDR/PCR process.

FIGS. **26**A–C show electropherogram results for an LDR/PCR process of ErbB, G6PD, Int2, p53, and SOD gene segments from normal human female DNA and from DNA of the breast cancer cell line ZR-75-30 and the gastric carcinoma cell line SKGT-2. The ErbB gene is known to be amplified in the cancer cell lines. Target-specific ligation product sequences of 104 bp are generated in 10 cycles (94° C. for 30 sec, 65° C. for 4 min) of LDR using 500 femtomoles of each ligation primer, 50 ng of genomic DNA, 124 units of *Thermus thermophillus* ("Tth") ligase, 2 μl of 10×buffer (0.2 M Tris, pH 8.5 and 0.1 M $MgCl_2$), 2 μl of 10 mM NAD, and 1 μl of 200 mM DTT in a volume of 20 μl. The ligation products are proportionally amplified in 26 cycles (94° C. for 15 sec, 60° C. for 50 sec) of PCR by the addition of 30 μl of a solution containing 5 μl 10×Stoffel buffer (Perkin Elmer), 25 picomoles of each oligonucleotide

primer, 2.5 units of Taq polymerase Stoffel fragment, and 8 μl of a solution 5 mM in each dNTP. After amplification, the products are digested with HaeIII and HinP1I to generate FAM-labeled products of 58 bp (ErbB (i.e. HER-2/neu/erbB oncogene)), 61 bp (G6PD), 67 bp (Int2 (i.e. int-2 oncogene)), 70 bp (p53) and 76 bp (SOD). These products are separated and analyzed on a 373A DNA sequencer using the Genescan 672 software package (Applied Biosystems, Inc., Foster City, Calif.). Results are displayed as electropherograms such that peak heights and areas reflect the amounts of the PCR products. In FIG. **26**A, the gene dosage determination for the five loci in normal human female DNA is shown. The peak heights and areas for G6PD, Int2, p53, and SOD are very similar. The peak height and area for ErbB is consistently small in normal genomic DNA. In FIG. **26**B, the peak height and area for ErbB are elevated when gene dosage is investigated in ZR-75-30, a cell line with known ErbB amplification. In FIG. **26**C, the gastric cell line, SKGT-2 shows dramatic amplification of the ErbB gene and a modest amplification of Int2. The G6PD gene peak may be embedded in the large ErbB peak.

FIGS. **27**A–C show electropherogram results for an LDR/PCR process to determine whether amplification of ErbB affected the relative peak heights of the other LDR oligonucleotide probes and PCR oligonucleotide primers for G6PD, Int2, p53, and SOD. In FIG. **27**A, the gene dosage determination for the four loci in normal human female DNA is shown. Peak heights and areas for G6PD, Int2, p53, and SOD are similar, as they were in the experiment using all five LDR primers. In FIG. **27**B, G6PD, Int2, and SOD analyzed in the ZR-75-30 breast cancer cell line show similar relative peak heights, comparable to their appearance in normal female DNA. The peak height for p53 is reduced, suggesting the deletion of this gene in a portion of the cells in this cell line. In FIG. **27**C, in the gastric carcinoma cell line, SKGT-2, G6PD, and p53 show comparable peak heights. The Int2 peak height remains relatively high, as it was in the experiment using all five LDR oligonucleotide probes. Thus, the LDR and PCR amplification of each product appears to be independent of the other products during the course of the experiment.

FIGS. **28**A–C show electropherogram results for the PCR phase of a primary PCR/secondary PCR/LDR process. Multiplex PCR amplification of 12 loci using primary PCR oligonucleotide primers produces approximately equal amounts of product. Over 80 gene regions with single-base polymorphisms were identified from the Human Genome Database. Twelve of these (see Table 10 and FIGS. **29**A–H) were amplified in a primary PCR phase as follows: Long primary PCR oligonucleotide primers were designed to have gene-specific 3' ends and 5' ends complementary to one of two sets of secondary PCR oligonucleotide primers. The upstream primary PCR oligonucleotide primers were synthesized with either FAM (i.e. 6-carboxyfluorescein; fluorescent dye used in sequencing and mutation detection) or TET (i.e. tetrachlorinated-6-carboxyfluorescein; fluorescent dye used in sequencing/mutation detection) fluorescent labels. All 24 base long primary PCR oligonucleotide primers were used at low concentration (2 picomole of each primer in 20 μl) in a 15 cycle primary PCR phase. After this, the two sets of secondary PCR oligonucleotide primers were added at higher concentrations (25 picomoles of each) and the secondary PCR phase was conducted for an additional 25 cycles. The products were separated on a 373 DNA Sequencer (Applied Biosystems). Panel A shows the electropherogram results for the FAM- and TET-labelled products combined. Panel B shows the FAM-labelled products alone. Panel C shows the TET-labelled products alone. The process produces similar amounts of multiplexed products without the need to adjust carefully primer concentrations or PCR conditions.

FIGS. **29**A–H show electropherogram results for the LDR phase of a primary PCR/secondary PCR/LDR process in detecting 12 biallelic genes for forensic identification. The primary and secondary PCR phases for the 12 polymorphic genes were performed as described in FIG. **28**A–C. However, the secondary PCR oligonucleotide primers were not fluorescently labelled. The secondary PCR process extension products were diluted into a ligase buffer containing 36 LDR oligonucleotide probes (one common and two discriminating primers for each locus). LDR oligonucleotide probe sets were designed in two ways: (i) allele-specific oligonucleotide probes were of the same length but contained either the FAM or TET label; or (ii) the allele-specific oligonucleotide probes were both labelled with HEX (i.e. hexachlorinated-6-carboxyfluorescein; fluorescent dye used in sequencing and mutation detection) but differed in length by two basepairs. After 20 cycles of the LDR phase, the ligation product sequences were resolved using a 10% polyacrylamide sequencing gel on a 373 DNA Sequencer (Applied Biosystems). Panel A and E show the 12 loci PCR/LDR profiles of two individuals. Panels B, C, and D show, respectively, the FAM, TET, and HEX data for the individual in panel A. Panels F, G, and H show, respectively, the FAM, TET, and HEX data for the individual in panel E. The individual in panel A is homozygous only at locus 6 (ALDOB (i.e. aldolase B)) and locus 8 (IGF (i.e. insulin growth factor)). The individual in panel E is heterozygous only at loci 3 (C6 (i.e. complement component C6)), 5 (NF1 (i.e. neurofibromatosis)), 6 (ALDOB), and 8 (IGF). This demonstrates that the primary PCR/primary PCR/LDR process can simultaneously distinguish both homozygous and heterozygous genotypes at multiple positions.

DETAILED DESCRIPTION OF THE INVENTION

I. Primary PCR/Secondary PCR/LDR Process

One aspect of the present invention relates to a method for identifying two or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. This method involves a first polymerase chain reaction phase, a second polymerase chain reaction phase, and a ligase detection reaction phase. This process involves analyzing a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences.

In the first polymerase chain reaction phase, one or more primary oligonucleotide primer groups are provided. Each group comprises one or more primary oligonucleotide primer sets with each set having a first oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion, and a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion. The first oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion and the second oligonucleotide primers of each set in the same group contain the same 5' upstream primer-specific portion. The oligonucleotide primers in a particular set are suitable for hybridization on complementary strands of a corresponding target nucleotide sequence to permit formation of a polymerase chain reaction product. However, there is a mismatch which interferes with formation of such a poly-

merase chain reaction product when the primary oligonucleotide primers hybridize to any other nucleotide sequence present in the sample. The polymerase chain reaction products in a particular set may be distinguished from other polymerase chain reaction products in the same group or groups. The primary oligonucleotide primers, the sample, and the polymerase are blended to form a primary polymerase chain reaction mixture.

The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as substantially described above. During hybridization, target-specific portions of the primary oligonucleotide primers hybridize to the target nucleotide sequences. The extension treatment causes hybridized primary oligonucleotide primers to be extended to form primary extension products complementary to the target nucleotide sequence to which the primary oligonucleotide primers are hybridized.

Although the upstream secondary primer-specific portions of a primary oligonucleotide primer set are not present on the target DNA, their sequences are copied by the second and subsequent cycles of the primary polymerase chain reaction phase. As a result, the primary extension products produced after the second cycle have the secondary primer-specific portions on their 5' ends and the complement of the primer-specific portion on their 3' ends.

Next, there is a second polymerase chain reaction phase. This phase involves providing one or a plurality of secondary oligonucleotide primer sets. Each set has a first secondary oligonucleotide primer containing the same sequence as the 5' upstream portion of the first primary oligonucleotide primer, and a second secondary oligonucleotide primer containing the same sequence as the 5' upstream portion of the second primary oligonucleotide primer from the same primary oligonucleotide primer set as the first primary oligonucleotide complementary to the first secondary primer. A set of secondary oligonucleotide primers may be used to amplify all of the primary extension products in a given group. The secondary oligonucleotide primers are blended with the primary extension products and the polymerase to form a secondary polymerase chain reaction mixture.

The secondary polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles having a denaturation treatment, a hybridization treatment, and an extension treatment, as substantially set forth above. During the hybridization treatment, the secondary oligonucleotide primers hybridize to complementary sequences present on the primary extension products but not to the original target sequence. The extension treatment causes the hybridized secondary oligonucleotide primers to be extended to form secondary extension products complementary to the primary extension products.

The last phase of this aspect of the present invention involves a ligase detection reaction process. Here, a plurality of oligonucleotide probe sets are provided where each set has a first oligonucleotide probe, having a secondary extension product-specific portion and a detectable reporter label, and a second oligonucleotide probe, having a secondary extension product-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a complementary secondary extension product-specific portion. However, there is a mismatch which interferes with such ligation when the oligonucleotide probes are hybridized to any other nucleotide sequence present in the sample. The ligation product of oligonucleotide probes in a particular set may be distinguished from either individual probes or other ligation products. The plurality of oligonucleotide probe sets, the secondary extension products, and a ligase are blended to form a ligase detection reaction mixture.

The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles having a denaturation treatment and hybridization treatment substantially as described above. In the hybridization treatment, the oligonucleotide probe sets hybridize at adjacent positions in a base-specific manner to the respective secondary extension products if present. As a result, adjacent probes ligate to one another to form a ligation product sequence containing the detectable reporter label and the secondary extension product-specific portions connected together. The oligonucleotide probe sets may hybridize to nucleotide sequences other than the respective complementary secondary extension products but do not ligate together due a presence of one or more mismatches and individually separate during the denaturation treatment. Following the ligation detection reaction cycles, the reporter labels of the ligation product sequences are detected which indicates the presence of one or more target nucleotide sequences in the sample.

FIGS. **1**, **2**, and **3** show flow diagrams of the primary PCR/secondary PCR/LDR process of the present invention utilizing either of two detection procedures. One alternative involves use of capillary electrophoresis or gel electrophoresis and a fluorescent quantification procedure. Alternatively, detection can be carried out by capture on an array of capture oligonucleotide addresses and fluorescent quantification. FIG. **1** relates to detection of germline mutations (e.g., a point mutation), while FIG. **2** detects biallelic polymorphisms, and FIG. **3** shows the detection of cancer-associated mutations.

FIG. **1** depicts the detection of a germline point mutation. In step 1, after DNA sample preparation, multiple exons are subjected to primary PCR amplification using Taq (i.e. *Thermus aquaticus*) polymerase under hot start conditions with oligonucleotide primers having a target-specific portion and a secondary primer-specific portion. At the end of the primary PCR phase, Taq polymerase may be inactivated by heating at 100° C. for 10 min or by a freeze/thaw step. The products of the primary PCR amplification phase are then subjected in step 2 to secondary PCR amplification using Taq polymerase under hot start conditions with the secondary oligonucleotide primers. At the end of the secondary PCR phase, Taq polymerase may be inactivated by heating at 100° C. for 10 min or by a freeze/thaw step. In step 3, products of the secondary PCR phase are then diluted 20-fold into fresh LDR buffer containing LDR oligonucleotide probes containing allele-specific portions and common portions. Step 4 involves the LDR phase of the process which is initiated by addition of Taq ligase under hot start conditions. During LDR, oligonucleotide probes ligate to their adjacent oligonucleotide probes only in the presence of target sequence which gives perfect complementarity at the junction site.

The products may be detected in two different formats. In the first format 5a, fluorescently-labeled LDR probes contain different length poly A or hexaethylene oxide tails. Thus, each ligation product sequence (resulting from ligation of two probes hybridized on normal DNA) will have a slightly different length and mobility such that several ligation product sequences yield a ladder of peaks. Thus, each ligation product (resulting from ligation of two probes hybridized on normal DNA) will have a slightly different length and mobility such that several ligation product

sequences yield a ladder of peaks. A germline mutation would generate a new peak on the electropherogram. Alternatively, the LDR probes may be designed such that the germline mutation ligation product sequence migrates with the same mobility as a normal DNA ligation product sequence, but it is distinguished by a different fluorescent reporter. The size of the new peak will approximate the amount of the mutation present in the original sample; 0% for homozygous normal, 50% for heterozygous carrier, or 100% for homozygous mutant. In the second format 5b, each allele-specific probe contains e.g., 24 additional nucleotide bases on their 5' ends. These sequences are unique addressable sequences which will specifically hybridize to their complementary address sequences on an addressable array. In the LDR reaction, each allele-specific probe can ligate to its adjacent fluorescently labeled common probe in the presence of the corresponding target sequence. Ligation product sequences corresponding to wild type and mutant alleles are captured on adjacent addresses on the array. Unreacted probes are washed away. The black dots indicate 100% signal for the wild type allele. The white dots indicate 0% signal for the mutant alleles. The shaded dots indicate the one position of germline mutation, 50% signal for each allele.

FIG. **2** depicts the detection of biallelic polymorphisms. In step 1, after DNA sample preparation, multiple exons are subjected to primary PCR amplification using Taq polymerase under hot start conditions with oligonucleotide primers having a target-specific portion and a secondary primer-specific portion. The products of the primary PCR amplification phase are then subjected in step 2 to secondary PCR amplification using Taq polymerase under hot start conditions with the secondary oligonucleotide primers. At the end of the secondary PCR phase, Taq polymerase may be inactivated by heating at 100° C. for 10 min or by a freeze/thaw step. In step 3, products of the secondary PCR phase are then diluted 20-fold into fresh LDR buffer containing LDR oligonucleotide probes containing allele-specific portions and common portions. Step 4 involves the LDR phase of the process which is initiated by addition of Taq ligase under hot start conditions. During LDR, oligonucleotide probes ligate to their adjacent oligonucleotide probes only in the presence of target sequence which gives perfect complementarity at the junction site. In the first format 5a, fluorescently-labeled LDR probes contain different length poly A or hexaethylene oxide tails. Each ligation product sequence will have a slightly different length and mobility, such that several LDR products yield a ladder of peaks. Alternatively, the LDR probes may be designed such that the ligation products for polymorphic alleles migrate at the same position but are distinguished by different fluorescent reporter groups. The size of the peaks will approximate the amount of each allele. In the second format 5b, each oligonucleotide probe contains unique addressable sequences with e.g., 24 additional nucleotide bases on their 5' ends. These sequences will specifically hybridize to their complementary address sequences on an array of capture oligonucleotides. In the LDR phase, each allele-specific probe can ligate to its adjacent fluorescently labeled common probe in the presence of corresponding target sequence. Ligation product sequences corresponding to each allele are captured on the array, while unligated oligonucleotide probes are washed away. The black dots indicate that both chromosomes have a given allele, the white dots show that neither chromosome has that allele, and the shaded dots indicate that one chromosome has a given allele.

FIG. **3** depicts the detection of cancer-associated mutation. In step 1, after DNA sample preparation, multiple exons are subjected to primary PCR amplification using Taq polymerase under hot start conditions with oligonucleotide primers having a target-specific portion and a secondary primer-specific portion. The products of the primary PCR amplification phase are then subjected in step 2 to secondary PCR amplification using Taq polymerase under hot start conditions with the secondary oligonucleotide primers. At the end of the secondary PCR phase, Taq polymerase may be inactivated by heating at 100° C. for 10 min or by a freeze/thaw step. Fluorescent quantification of PCR products can be achieved using capillary or gel electrophoresis in step 3. In step 4, the products are spiked with a 1/100 dilution of marker DNA (for each of the fragments). This DNA is homologous to wild type DNA, except it contains a mutation which is not observed in cancer cells, but which may be readily detected with the appropriate LDR probes. In step 5, the mixed DNA products in products of the secondary PCR phase are then diluted 20-fold into fresh LDR buffer containing LDR oligonucleotide probes containing allele-specific portions and common portions. Step 6 involves the LDR phase of the process which is initiated by addition of Taq ligase under hot start conditions. During LDR, oligonucleotide probes ligate to their adjacent oligonucleotide probes only in the presence of target sequence which gives perfect complementarity at the junction site.

The products may be detected in the same two formats discussed above. In the format of step 7a, products are separated by capillary or gel electrophoresis, and fluorescent signals are quantified. Ratios of mutant peaks to marker peaks give the approximate amount of cancer mutations present in the original sample divided by 100. In the format of step 7b, products are detected by specific hybridization to complementary sequences on an addressable array. Ratios of fluorescent signals in mutant dots to marker dots give the approximate amount of cancer mutations present in the original sample divided by 100.

As shown in FIG. **4**, two DNA fragments of interest are treated with the primary PCR/secondary PCR/LDR process of the present invention. Initially, the double stranded DNA molecules are denatured to separate the strands. This is achieved by heating to a temperature of 80–105° C. Low concentrations of primary PCR oligonucleotide primers, containing a 3' target-specific portion (shaded area) and 5' secondary primer-specific portion (black area), are then added and allowed to hybridize to the strands, typically at a temperature of 50–85° C. A thermostable polymerase (e.g., Taq *aquaticus* polymerase) is also added, and the temperature is then adjusted to 50–85° C. to extend the primer along the length of the nucleic acid to which the primer is hybridized. After the extension phase of the polymerase chain reaction, the resulting double stranded molecule is heated to a temperature of 80–105° C. to denature the molecule and to separate the strands. These hybridization, extension, and denaturation steps may be repeated a number of times to amplify the target to an appropriate level.

In the secondary PCR phase, the products of the primary PCR phase are blended with secondary PCR oligonucleotide primers and allowed to hybridize to one another, typically at a temperature of 50–85° C. The secondary oligonucleotide primers are usually used in higher concentrations than are the primary oligonucleotide primers. Taq polymerase is also added, and the temperature is then adjusted to 50–85° C. to extend the primer along the length of the primary PCR extension products to which the secondary oligonucleotide primer is hybridized. After the extension phase of the polymerase chain reaction, the resulting double stranded molecule is heated to a temperature of 80–105° C. to

denature the molecule and to separate the strands. These hybridization, extension, and denaturation steps may be repeated a number of times to amplify the target to an appropriate level.

Once the secondary PCR phase of the process is completed, the ligation detection reaction phase begins, as shown in FIG. **4**. After denaturation of the target nucleic acid, if present as a double stranded DNA molecule, at a temperature of 80–105° C., preferably 94° C., ligation detection reaction oligonucleotide probes for one strand of the target nucleotide sequence are added along with a ligase (for example, as shown in FIG. **4**, a thermostable ligase like *Thermus aquaticus* ligase). The oligonucleotide probes are then allowed to hybridize to the target nucleic acid molecule and ligate together, typically, at a temperature of 45–85° C., preferably, 65° C. When there is perfect complementarity at the ligation junction, the oligonucleotides can be ligated together. Where the variable nucleotide is T or A, the presence of T in the target nucleotide sequence will cause the oligonucleotide probe with the F1 reporter label to ligate to the common oligonucleotide probe with the 5' poly A tail A_n, and the presence of A in the target nucleotide sequence will cause the oligonucleotide probe with the F2 reporter label to ligate to the common oligonucleotide probe with A_n. Similarly, where the variable nucleotide is A or G, the presence of T in the target nucleotide sequence will cause the oligonucleotide probe with F3AA reporter label (i.e. the F3 reporter label coupled to 2 additional bases forming a 5' poly A spacer) to ligate to the common oligonucleotide probe with the 5' poly A tail A_{n+4}, and the presence of C in the target nucleotide sequence will cause the oligonucleotide probe with the F3 reporter label to ligate to the common oligonucleotide probe with the 5' poly A tail A_{n+4}. Following ligation, the material is again subjected to denaturation to separate the hybridized strands. The hybridization/ligation and denaturation steps can be carried out through one or more cycles (e.g., 1 to 50 cycles) to amplify target signals. Equimolar ligation of both F3-labeled oligonucleotides indicates the individual is heterozygous for that locus, whereas ligation of only the F2 labeled oligonucleotides indicates the individual is homozygous for the other locus.

In FIG. **4**, the poly A_n and poly A_{n+4} tails are used where quantification is to be carried out by capillary or gel electrophoresis. The tails of differing length cause the corresponding different ligation product sequences to form bands at different locations in the gel or capillary. The presence of these bands at different locations permit the corresponding nucleotide differences in the DNA being analyzed to be identified. Although ligation product sequences can be distinguished based on the use of different reporter labels, the combination of different reporter labels and different length tails permits greater numbers of nucleotide differences to be distinguished. This is important for multiplex detection processes.

As noted above with respect to FIGS. **1** to **3**, detection can be carried out on an addressable array instead of with gel or capillary electrophoresis. The use of such addressable arrays require that the poly A tails on the LDR oligonucleotide probe not containing a reporter label (i.e. tails A_n and A_{n+4}) be replaced with different addressable array-specific oligonucleotide portions. As explained more fully infra, a solid support is provided with an array of capture oligonucleotides, some of which are complementary to the different addressable array-specific oligonucleotide portions. Hybridization of these portions to their complementary capture oligonucleotide probes indicates the presence of a corresponding nucleotide difference.

FIG. **5** is a schematic diagram of a primary PCR/secondary PCR/LDR process, in accordance with the present invention, where any possible base in 2 DNA molecules of interest are distinguished. The primary and secondary PCR processes are carried out in substantially the same way as described for FIG. **4**. Appearance of fluorescent reporter labels F1, F2, F3, and F4 in conjunction with the left hand DNA molecule indicates the presence of the A, G, C, and T alleles in the DNA molecule, respectively. As shown in FIG. **5**, equal amounts of the F1 and F3 reporter labels indicates that the individual in question is heterozygous for the A and C alleles. With respect to analysis of the right hand DNA molecule in FIG. **5**, the same reporter label is used to indicate the presence of the different alleles; however, on each oligonucleotide probe with the distinguishing bases, there are different 5' poly A tails. More particularly, a 2 unit poly A tail, a 4 unit poly A tail, a 6 unit poly A tail, and an 8 unit poly A tail correspond to the T, C, G, and A alleles in the DNA molecule, respectively. As shown in FIG. **5**, equal amounts of the F1 reporter label with the A_6 and A_4 tails indicates that the individual in question is heterozygous for the G and C alleles.

FIG. **6** is a schematic diagram of a primary PCR/secondary PCR/LDR process, in accordance with the present invention, for detecting the presence of any possible base at two nearby sites in DNA molecules of interest. The primary and secondary PCR phases are carried out in substantially the same way as described for FIG. **4**. Here, the LDR probes are able to overlap, yet are still capable of ligating provided there is perfect complementarity at the junction. This distinguishes LDR from other approaches, such as allele-specific PCR where overlapping primers would interfere with one another. In FIG. **6**, the discriminating oligonucleotide probes contain the reporter label with the discriminating base on the 3' end of these probes. The poly A tails are on the 3' end of common oligonucleotide probes. In the left hand DNA molecule, the presence of equal amounts of ligation product sequences with reporter labels F1 and F3 shows that the individual in question is heterozygous for the A and C alleles in the first position. Similarly, in the second position for the left hand DNA molecule, the presence of ligation product sequences with reporter labels F2, F3, and F4 shows that the individual in question is heterozygous for the G, C, and T alleles. Turning to the right hand DNA molecule, the presence of equal amounts of ligation product sequences with reporter label F1 having the A_6 and A_4 tails indicates that at the first position, the individual in question is heterozygous for the G and C alleles. In the second position for the right hand DNA molecule, the presence of the equal amounts of ligation product sequences with reporter label F1 having the A_8 and A_2 tails indicates that the individual in question is heterozygous for the A and T alleles.

FIG. **7** is a schematic diagram depicting the use of the primary PCR/secondary PCR/LDR process of the present invention to detect a low abundance mutation in the presence of an excess of normal sequence. Here, in the left hand DNA molecule is codon 12 of the K-ras gene, sequence GGT, which codes for glycine ("Gly"). A small percentage of the cells contain the G to A mutation in GAT, which codes for aspartic acid ("Asp"). The LDR probes for wild-type (i.e. normal) sequences are missing from the reaction. If the normal LDR probes (with the discriminating base being G) were included, they would ligate to the common probes and overwhelm any signal coming from the mutant target. Instead, as shown in FIG. **7**, the existence of a ligation product sequence with fluorescent label F1 and the A_{n+2} tail

indicates the presence of the aspartic acid encoding mutant. In the right hand DNA molecule, FIG. **7** shows codon 61 of the K-ras gene sequence CAG which codes for glutamine ("Gln"). A small percentage of the cells contain the C to G mutation in GAG, which codes for glutamic acid ("Glu"). Again, the LDR oligonucleotide probes do not include the C and A bases found in the wild type form to avoid overwhelming the mutant signal. For this DNA molecule, the existence of a ligation product sequence with fluorescent label F2 and the A_{n+4} tail indicates the presence of the glutamic acid encoding mutant.

II. LDR/PCR Process

A second aspect of the present invention relates to a method for identifying one or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. This method has a ligase detection reaction phase followed by a polymerase chain reaction phase. This method involves providing a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences.

In the ligase detection reaction phase, one or more of oligonucleotide probe sets are provided. Each set has a first oligonucleotide probe, having a target-specific portion and a 5' upstream primer-specific portion, and a second oligonucleotide probe, having a target-specific portion and a 3' downstream primer-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target nucleotide sequence. However, there is a mismatch which interferes with such ligation when they are hybridized to any other nucleotide sequence present in the sample. The sample, the plurality of oligonucleotide probe sets, and a ligase are blended together to form a ligase detection reaction mixture.

The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles. These cycles include a denaturation treatment and a hybridization treatment. In the denaturation treatment, any hybridized oligonucleotides are separated from the target nucleotide sequences. The hybridization treatment causes the oligonucleotide probe sets to hybridize at adjacent positions in a base-specific manner to their respective target nucleotide sequences if present in the sample. Once hybridized, the oligonucleotide probe sets ligate to one another to form a ligation product sequence. This product contains the 5' upstream primer-specific portion, the target-specific portions connected together, and the 3' downstream primer-specific portion. The ligation product sequence for each set is distinguishable from other nucleic acids in the ligase detection reaction mixture. The oligonucleotide probe sets hybridized to nucleotide sequences in the sample other than their respective target nucleotide sequences but do not ligate together due to a presence of one or more mismatches and individually separate during the denaturation treatment.

In the polymerase chain reaction, one or a plurality of oligonucleotide primer sets are provided. Each set has an upstream primer containing the same sequence as the 5' upstream primer-specific portion of the ligation product sequence and a downstream primer complementary to the 3' downstream primer-specific portion of the ligation product sequence, where one primer has a detectable reporter label. The ligase detection reaction mixture is blended with the one or a plurality of oligonucleotide primer sets and the polymerase to form a polymerase chain reaction mixture.

The polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles which include a denaturation treatment, a hybridization treatment, and an extension treatment. During the denaturation treatment, hybridized nucleic acid sequences are separated. The hybridization treatment causes primers to hybridize to their complementary primer-specific portions of the ligation product sequence. During the extension treatment, hybridized primers are extended to form extension products complementary to the sequences to which the primers are hybridized. In a first cycle of the polymerase chain reaction phase, the downstream primer hybridizes to the 3' downstream primer-specific portion of the ligation product sequence and is extended to form an extension product complementary to the ligation product sequence. In subsequent cycles, the upstream primer hybridizes to the 5' upstream primer-specific portion of the extension product complementary to the ligation product sequence and the 3' downstream primer hybridizes to the 3' downstream portion of the ligation product sequence.

Following the polymerase chain reaction phase of this process, the reporter labels are detected and the extension products are distinguished to indicate the presence of one or more target nucleotide sequences in the sample.

FIG. **8** is a flow diagram depicting the LDR/PCR process of the present invention with or without restriction endonuclease digestion and using capillary electrophoresis detection. In step 1, a DNA sample is mixed with Taq ligase and oligonucleotide probes containing a target-specific portion and a primer-specific portion. The mixture is subjected to an LDR process to produce ligation product sequences containing the ligated target-specific portions and the primer-specific portions. Step 2 involves mixing the ligation product sequences with Taq polymerase and primers and subjecting the mixture to a PCR process. The next step is determined as a function of whether the ligation product sequences are the same or different sizes. Where the ligation product sequences are different sizes, step 3a is selected which involves subjecting the extension products from PCR to capillary electrophoresis or gel electrophoresis, either of which is followed by fluorescent quantification. Step 3b is utilized where the ligation product sequences are the same size and involves subjecting the extension products from the PCR phase to restriction endonuclease digestion. This generates digestion fragments of unique size which can be subjected to capillary electrophoresis or gel electrophoresis, followed by fluorescent quantification, according to step 4b. When step 3a is selected, the curve generated as a result of electrophoresis shows three ligation product sequences migrating at lengths of 104, 107, and 110, with the peak areas representing amplification of the Her-2 gene, loss of heterozygosity of the p53 gene, and the control SOD gene, respectively. The electrophoresis curve where steps 3b and 4b are used involves three ligation product sequence restriction fragments at lengths of 58, 70, and 76, with the peak areas representing amplification of the Her-2 gene, loss of heterozygosity of the p53 gene, and the control SOD gene, respectively.

As an alternative to FIG. **8**, FIG. **9** shows the LDR/PCR process of the present invention where, in step 3, the extension products are captured on an array of capture oligonucleotide addresses. The capture oligonucleotide probes can be complementary to a nucleotide sequence across the ligation junction. The number of gene copies captured on the array of capture oligonucleotides is then determined by fluorescent quantification as compared with known controls. In FIG. **8**, such analysis of the array indicates ligation product sequences hybridizing to gene-specific addresses, where the fluorescent intensity represents

amplification of the Her-2 gene, loss of heterozygosity of the p53 gene, and the control SOD gene, respectively.

FIG. **10** is a schematic diagram depicting an LDR/PCR process for multiplex detection of gene amplifications and deletions. Here, the ratio of the Her-2/neu gene from Chromosome 17q, the p53 gene from Chromosome 17p, and the SOD gene from Chromosome 21q is detected. Following denaturation of DNA at 94° C., pairs of oligonucleotide probes, having a target-specific portion and a primer-specific portion, are allowed to anneal adjacent to each other on target nucleic acids and ligate to one another (in the absence of mismatches). This ligase detection reaction is carried out with Tth ligase at a hybridization/ligation temperature of 65° C. which is well below the T_m values of 75° C. for the oligonucleotide probes. Next, the ligation product sequences are simultaneously amplified by PCR using Taq polymerase and two common primers complementary to the primer-specific portion, one of which is fluorescently labeled. This maintains the proportionality of the target sequences initially present in the sample. The extension products are then digested with HaeIII and Hinp1I which releases fluorescently labeled fragments of unique sizes for each target sequence present in the sample. The digestion products are separated and analyzed on an Applied Biosystems, Inc. (Foster City, Calif.) 373A DNA Sequencer. The peak heights and areas are related to the relative copies of genes present in the initial target sample.

FIG. **11** is a schematic diagram, depicting a problem which can be encountered with the allele-specific LDR/PCR process. While a PCR/LDR process is very powerful, there may be circumstances where a multiplexed allele-specific LDR/PCR process of the present invention would be preferred. The concept is to have one or more sets of LDR oligonucleotide probes, each set characterized by (a) a first oligonucleotide probe, having a target-specific portion and a 5' upstream primer-specific portion, and (b) a second oligonucleotide probe, having a target-specific portion and a 3' downstream primer-specific portion. As shown in step 1 of FIG. **11**, the LDR oligonucleotide probes anneal adjacent to each other on the target sequence. An LDR reaction using thermostable ligase (black dot) would form a ligation product sequence provided there is perfect complementarity at the ligation junction. In step 2, the ligation product sequences are PCR amplified with primer sets, each set characterized by (a) an upstream primer containing the same sequence as the 5' upstream primer-specific portion of a ligation product sequence and (b) a downstream primer complementary to the 3' downstream primer-specific portion of that ligation product sequence. The primers are shown as black lines in step 2. If one primer is fluorescently labeled, it will generate a fluorescent product which may be detected in a variety of detection schemes. For the LDR/PCR process to be specific, a PCR extension product should not be formed in the absence of a ligation event. Unfortunately, the possibility exists for polymerase to extend the first LDR oligonucleotide probe (off normal target), forming a product containing the length of the target sequence, and a primer-specific portion on the 5' end. Meanwhile, polymerase can make several complementary copies of the downstream LDR probe using the downstream primer. In a second amplification cycle, this downstream LDR probe extension product can anneal to the upstream LDR probe extension product off the target sequence, and generate a sequence containing the target region flanked by the two primer-specific sequences. This product will amplify as the LDR product, and thus yield a false positive signal.

FIG. **12** is a schematic drawing showing a solution to the allele specific LDR/PCR problem, utilizing an intermediate exonuclease digestion step. Allele-specific LDR/PCR can be achieved while significantly reducing background ligation independent (incorrect) target amplification. To do so, it is necessary to eliminate one or more of the components required for ligation independent PCR amplification, without removing the information content of the ligation product sequence. One solution is to use exonuclease in step 2 to digest unreacted LDR oligonucleotide probes from step 1. By blocking the end which is not ligated, for example the 3' end of the downstream oligonucleotide probe, one probe can be made substantially resistant to digestion, while the other is sensitive. Only the presence of full length ligation product sequence will prevent digestion of the upstream primer. Blocking groups include use of a thiophosphate group and/or use of 2-O-methyl ribose sugar groups in the backbone. Exonucleases include Exo I (3'–5'), Exo III (3'–5'), and Exo IV (both 5'–3' and 3'–5'), the later requiring blocking on both sides. One convenient way to block both probes is by using one long "padlock" probe (see M. Nilsson et. al., "Padlock Probes: Circularizing Oligonucleotides for Localized DNA Detection," *Science* 265:2085–88 (1994), which is hereby incorporated by reference), although this is by no means required. An advantage of using exonucleases, for example a combination of Exo I (single strand specific) and Exo III (double strand specific), is the ability to destroy both target and one LDR probe, while leaving the ligation product sequences substantially undigested. By using an exonuclease treatment prior to PCR, in accordance with steps 3 and 4, either one or both oligonucleotide probes in each set are substantially reduced, and thus hybridization of the remaining oligonucleotide probes to the original target DNA (which is also substantially reduced by exonuclease treatment) and formation of a ligation product sequence which is a suitable substrate for PCR amplification by the oligonucleotide primer set is substantially reduced. In other words, formation of ligation independent labeled extension products is substantially reduced or eliminated.

FIG. **13** is a flow diagram showing an allele-specific LDR/PCR process using exonuclease digestion with either size based- or DNA array based-detection. The flow diagram shows the three reactions required for the multiplexed allele-specific LDR/PCR process. In step 1, sets of LDR oligonucleotide probes (wherein the downstream probes are blocked on their 3' ends) are ligated in the presence of the correct allele target using Taq DNA ligase. Unreacted upstream probes are digested with exonuclease in step 2, coincidentally, target is also digested. Finally, in step 3, primer sets are used to amplify the ligation product sequence by hybridizing to the primer specific portions of ligation product sequences. In step 4a, the LDR oligonucleotide probes in a given particular set generate a unique length product, and thus may be distinguished from either oligonucleotide probes or other ligation products. After the PCR reaction, the products are separated by size or electrophoretic mobility. Labels on the PCR primers are detected, and the products are distinguished by size. In step 4b, the LDR oligonucleotide probes in a particular set use may be distinguished from either oligonucleotide probes or other ligation product sequences by differences in the sequences of the PCR primers. By using a plurality of oligonucleotide primer sets, each set characterized by (a) an upstream primer containing the same sequence as the 5' upstream primer-specific portion of a ligation product sequence, and (b) a downstream primer complementary to the 3' downstream primer-specific portion of that ligation product sequence, wherein one primer has a detectable reporter label and the other primer contains an addressable nucleotide sequence

linked to the 5' end of that primer such that the addressable nucleotide sequence remains single stranded after a PCR reaction, all the products can be distinguished. The latter may be achieved by using a non-natural base within a PCR primer which polymerase cannot extend through, thus generating PCR products which have single stranded tails. See C. Newton, et. al., "The Production of PCR Products with 5' Single-stranded Tails Using Primers that Incorporate Novel Phosphoramidite Intermediates," *Nucl. Acids Res.* 21(3): 1155–62 (1993), which is hereby incorporated by reference. By providing a DNA array with different capture oligonucleotides immobilized at different particular sites, where the capture oligonucleotides have nucleotide sequences complementary to the addressable nucleotide sequences on the primers, the PCR extension products can hybridize to the DNA array. Finally, the labels of extension product sequences captured using the addressable array-specific portions immobilized to the DNA array at particular sites can be detected. This indicates the presence of one or more target nucleotide sequences in the sample.

FIG. **14** is a flow diagram showing a quantitative allele-specific LDR/PCR process using exonuclease digestion in step 3 with either size based- or DNA array based-detection. The flow diagram shows how one can quantify the amounts of different targets (especially low abundance cancer mutations) by adding marker sequence(s) (step 1) at the start of the LDR reaction (step 2). In this embodiment, the biochemical reactions (i.e. PCR (step 4)) are followed, as described in FIG. **13**, and the relative amount of mutant product to marker product are quantified using capillary or gel electrophoresis (step 5a) or capture on an addressable array (step 5b). The amount of mutant target present in the original sample can then be determined.

FIG. **15** is a schematic drawing showing an allele-specific LDR/PCR process with exonuclease digestion (step 2) for detection of mutations or polymorphisms. Mutations and polymorphisms may be distinguished as described in FIG. **12**. In this example, in step 1, the upstream LDR oligonucleotide probes, which have the discriminating allele-specific base at the 3' end of the target-specific portion, have different 5' upstream primer-specific portions. Thus, different primers (in the PCR amplification step (i.e. step 3)) may be labeled with different fluorescent groups (Fam and Tet) to allow for distinction of products (step 4). An array based detection scheme may also be used, where the upstream (allele-specific) probes have different 5' upstream primer-specific portions, and the different PCR primers contain different addressable nucleotide sequences which remain single stranded after a PCR reaction.

FIG. **16** is a schematic drawing showing an allele-specific LDR (step 1)/PCR (step 3) process using exonuclease digestion (step 2) for detection of mononucleotide or dinucleotide repeat polymorphisms. One of the most powerful uses of LDR/PCR is for detecting nucleotide repeat polymorphisms, a task which cannot be achieved by allele-specific PCR (because the 3' nucleotide is always the same), nor easily achieved by observing PCR product size variation (due to Taq polymerase slippage during amplification). In FIG. **16**, the LDR oligonucleotide probes distinguish between an A_9 and A_{10} mononucleotide repeat sequence by virtue of the specificity of thermostable DNA ligase. LDR products are only formed on the correct length target sequence, and thus the presence of that target is distinguished (step 4).

FIG. **17** is schematic drawing showing an allele-specific LDR/PCR process using exonuclease digestion (step 2) for detection of low abundance mononucleotide or dinucleotide repeat mutations. Mononucleotide repeat length mutations may be distinguished as described in FIG. **12**. In FIG. **17**, the LDR oligonucleotide probes (step 1) distinguish between an A_8, A_9 (mutants), and A_{10} (normal) mononucleotide repeat sequences by virtue of the specificity of thermostable DNA ligase. The two upstream LDR oligonucleotide probes differ in the length of the mononucleotide sequence at their 3' ends of their target specific portion and have different 5' upstream primer-specific portions. Thus, different primers (in the PCR amplification step (step 3)) may be labeled with different fluorescent groups (Fam and Tet) to allow for distinction of products (step 4). This has the distinct advantage of allowing mononucleotide repeat polymorphisms to be distinguished based on fluorescent label instead of size, the latter being susceptible to false positives due to polymerase slippage. An array based detection scheme may also be used, where the upstream (allele-specific) probes have different 5' upstream primer-specific portions, and the different PCR primers contain different addressable nucleotide sequences which remain single stranded after a PCR reaction.

FIG. **18** is a flow diagram, showing an allele-specific LDR/PCR process using uracil N-glycosylase selection with either size based- or DNA array based-detection. The flow diagram shows the four reactions required for multiplexed allele-specific LDR/PCR. Sets of LDR oligonucleotide probes (wherein one or both probes contain deoxy-uracil in place of deoxythimidine) are ligated in the presence of the correct allele target using Taq DNA ligase in step 1. A complementary copy of the ligation product sequence is made with sequenase in step 2. Sequenase is a modified T7 polymerase, with any easily inactivated polymerase (i.e. mesophilic polymerases such as, *E. coli* polymerase) being useful. Both ligation product sequences and unreacted probes are destroyed with uracil N-glycosylase in step 3. The advantage of using uracil N-glycosylase is its proven ability in carry-over prevention for PCR. Finally PCR primer sets are used to amplify the sequenase extension products in step 4. In step 5a, the LDR oligonucleotide probes in a particular set generate a unique length product, and thus may be distinguished from either probes or other ligation products. After the PCR reaction, the products are separated by size or electrophoretic mobility. Labels on the PCR primers are detected, and products are distinguished by size. In step 5b, the LDR oligonucleotide probes in a particular set may be distinguished from either probes or other ligation product sequences by differences in the sequences of the primer-specific portions. By using a plurality of oligonucleotide primer sets, each set characterized by (a) an upstream primer containing the same sequence as the 5' upstream primer-specific portion of a ligation product sequence, and (b) a downstream primer complementary to the 3' downstream primer-specific portion of that ligation product sequence. One primer has a detectable reporter label, and the other primer contains an addressable array-specific portion linked to the 5' end of that primer such that the addressable array-specific portion remains single stranded after a PCR reaction, one can distinguish all the products. By providing a DNA array with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the addressable array-specific portions on the primers, the PCR extension products can be hybridized to the DNA array. Finally, the labels of extension product sequences captured using the addressable nucleotide sequence portions and immobilized to the DNA array at particular sites can be detected to indicate the presence of one or more target nucleotide sequences in the sample.

FIG. **19** is a flow diagram showing a quantitative allele-specific LDR/PCR process using uracil N-glycosylase selec-

tion with either size based- or DNA array based-detection. The flow diagram shows how one can quantify the amounts of different targets (especially low abundance cancer mutations) by adding marker sequence(s) in step 1 at the start of the LDR phase in step 2. As described in FIG. **18**, the biochemical reactions (i.e. sequenase treatment (step 3), uracil N-glycosylase selection (step 4), and PCR (step 5)) are preceded with, and the relative amount of mutant product to marker product is quantified using capillary or gel electrophoresis (step 6a) or capture on an addressable array (step 6b). From this information, the amount of mutant target present in the original sample can be determined.

FIG. **20** is a schematic drawing showing an allele-specific LDR/PCR process using uracil N-glycosylase selection (step 3) (after sequenase treatment (step 2)) for detection of mononucleotide or dinucleotide repeat polymorphisms. One of the most powerful uses of the LDR/PCR process is for detecting nucleotide repeat polymorphisms, a task which cannot be achieved by allele-specific PCR (since the 3' nucleotide is always the same), nor easily achieved by observing PCR product size variation (due to Taq polymerase slippage during amplification), as in step 4. In FIG. **20**, the LDR (step 1) oligonucleotide probes distinguish between an A_9 and A_{10} mononucleotide repeat sequence by virtue of the specificity of thermostable DNA ligase. Ligation product sequences are only formed on the correct length target sequence, and, thus, the presence of that target is distinguished in step 5.

FIG. **21** is a schematic drawing showing an allele-specific LDR/PCR process using uracil N-glycosylase selection for detection of low abundance mononucleotide or dinucleotide repeat mutations. Mononucleotide repeat length mutations may be distinguished as described in FIG. **18**. In FIG. **21**, the LDR oligonucleotide probes distinguish between an A_8, A_9 (mutants), and A_{10} (normal) mononucleotide repeat sequences by virtue of the specificity of thermostable DNA ligase (step 1). Sequenase treatment (step 2) and uracil N-glycosylase selection (step 3) are then carried out. The two upstream LDR oligonucleotide probes differ in the length of the mononucleotide sequence at the 3' ends of their target-specific portion, and have different 5' upstream primer-specific portions. Thus, different primers (in the PCR amplification step (steps 4–5)) may be labeled with different fluorescent groups (Fam and Tet) to allow for distinction of products. This has the distinct advantage of allowing one to distinguish mononucleotide repeat polymorphisms based on fluorescent label instead of size, the latter being susceptible to false positives due to polymerase slippage. An array based detection scheme may also be used, where the upstream (allele-specific) probes have different 5' upstream primer-specific portions, and the different PCR primers contain different addressable array-specific portions which remain single stranded after a PCR reaction.

The LDR/exonuclease/PCR process described with reference to FIGS. **11** to **17** and the LDR/sequenase/uracil N-glycosylase/PCR process set forth in FIGS. **18–21** provide the ability to multiplex detect and then PCR amplify many different target sequences and to distinguish multiple single-base or sequence variations, all in a single reaction tube. This is achieved by combining the sensitivity of PCR with the selectivity of LDR. Since the selection of mutant sequences is mediated by LDR rather than PCR, the primary PCR/secondary PCR/LDR process is less susceptible to false-positive signal generation. In addition, the primary PCR/secondary PCR/LDR process allows detection of closely-clustered mutations, detection of single base or small insertions and deletions in small repeat sequences, quantitative detection of less than 1% mutations in high background of normal DNA, and detection of ligation product sequences using addressable arrays Detection of single base or small insertions and deletions in small and medium repeat sequences may cause "stutter" when the primary amplification is PCR. No other currently-available technique can adequately solve this problem, especially when the target sequence containing the mononucleotide repeat polymorphism is present in a lower abundance than normal DNA. In fact, analysis of genomic mutations which involve repeat sequence changes is severely hampered by the PCR "stutter" problem. By using the LDR/PCR process of the present invention, it is possible to detect down to 1% mutations in a high background of normal DNA. The only relatively minor challenges presented by this process are that the mutations must be known and that 3 different enzymes/reaction conditions must be utilized.

III. Primary PCR/Secondary PCR Process

A third aspect of the present invention also involves a method for identifying two or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in one or more target nucleotide sequences. This method involves subjecting a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences to two successive polymerase chain reaction phases.

For the first polymerase chain reaction phase, one or more primary oligonucleotide primer groups are provided where each group comprises one or more primary oligonucleotide primer sets. Each set has a first oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primary-specific portion, and a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion. The first oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion and the second oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion. The oligonucleotide primers in a particular set are suitable for hybridization on complementary strands of a corresponding target-nucleotide sequence to permit formation of a polymerase chain reaction product. However, there is a mismatch which interferes with formation of such a polymerase chain reaction product when the primary oligonucleotide primers hybridize to any other nucleotide sequence present in the sample. The polymerase chain reaction products in a particular set may be distinguished from other polymerase chain reaction products with the same group or other groups. The primary oligonucleotide primers are blended with the sample and the polymerase to form a primary polymerase chain reaction mixture.

The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as described above. During the hybridization treatment, the target-specific portion of a primary oligonucleotide primer is hybridized to the target nucleotide sequences. In the extension treatment, the hybridized primary oligonucleotide primers are extended to form primary extension products complementary to the target nucleotide sequence to which the primary oligonucleotide primer is hybridized.

Although the upstream secondary primer-specific portions of a primary oligonucleotide primer set are not present on the target DNA, their sequences are copied by the second and subsequent cycles of the primary polymerase chain reaction phase. As a result, the primary extension products

produced after the second cycle have the secondary primer-specific portions on their 5' ends and the complement of primer-specific portion on their 3' ends.

In the second polymerase chain reaction phase of this aspect of the present invention, one or a plurality of secondary oligonucleotide primer sets are provided. Each set has a first secondary primer having a detectable reporter label and containing the same sequence as the 5' upstream portion of a first primary oligonucleotide primer, and a second secondary primer containing the same sequence as the 5' upstream primer of the second primary oligonucleotide primer from the same primary oligonucleotide primer set as the first primary oligonucleotide complementary to the first secondary primer. A set of secondary oligonucleotide primers amplify the primary extension products in a given group. The secondary oligonucleotide primers are blended with the primary extension products and the polymerase to form a secondary polymerase chain reaction mixture.

The secondary polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as described above. In the hybridization treatment, the secondary oligonucleotide primers are hybridized to the primary extension products, while the extension treatment causes the hybridized secondary oligonucleotide primers to be extended to form secondary extension products complementary to the primary extension products. After subjecting the secondary polymerase chain reaction mixture to the two or more polymerase chain reaction cycles, the labelled secondary extension products are detected. This indicates the presence of one or more target nucleotide sequences in the sample.

FIG. **22** is a flow diagram depicting a primary PCR/secondary PCR process, in accordance with the present invention, for detection of microsatellite repeats. In step 1 (i.e. the primary PCR phase), after DNA sample preparation, multiple exons are amplified using Taq polymerase under hot start conditions with oligonucleotide primers having a target-specific portion and a secondary primer-specific portion. Step 2 involves a secondary PCR phase where Taq polymerase is used to amplify the primary PCR extension products with oligonucleotide primers containing the same sequence as the secondary primer-specific portion of the primary PCR primers. The extension products resulting from the secondary PCR phase are subjected in step 3 to capillary electrophoresis or gel electrophoresis, followed by fluorescent quantification. The electrophoresis results in FIG. **22** indicate the presence of both alleles (i.e., chromosomes) containing RB1 and NM23 and loss of heterozygosity (i.e., loss of allele on one chromosome) for p53.

FIG. **23** is a schematic diagram depicting a primary PCR/secondary PCR process, according to the present invention, for detection of the loss of heterozygosity due to insertions and deletions in microsatellite repeats. The primary PCR phase in step 1 is initiated by denaturing the sample DNA at 94° C. Long PCR oligonucleotide primers, having 3' ends complementary to unique DNA surrounding microsatellite repeat sequences and 5' ends containing the same sequence as one of two primers utilized in the secondary PCR phase, are then caused to anneal to target DNA at 65° C. The primary PCR phase is carried out for 10–15 cycles. The long primers utilized in the primary PCR phase can be multiplexed as long as they do not amplify alleles with overlapping length ranges. These reactions must be carried out on tumor and corresponding normal DNA to identify informative (i.e heterozygous) loci. In step 2 (i.e secondary PCR amplification), primers complementary to the 5' ends of the primary PCR primers (one fluorescently labeled) are then used to amplify the primary PCR extension products at nearly equal efficiency. The secondary PCR extension products are then separated and analyzed by gel electrophoresis and an Applied Biosystems Inc. 373A DNA Sequencer using the Genescan 672 software package. Areas of loss of heterozygosity at informative loci are identified. The analysis in FIG. **23** shows the presence of both alleles (i.e., chromosomes) containing RB1 and NM23 and loss of heterozygosity (i.e., loss of allele on one chromosome) for p53.

IV. General Process Information

The ligase detection reaction is described generally in WO 90/17239 to Barany et al., F. Barany et al., "Cloning, Overexpression and Nucleotide Sequence of a Thermostable DNA Ligase-encoding Gene," *Gene,* 109:1–11 (1991), and F. Barany, "Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase," *Proc. Natl. Acad. Sci. USA,* 88:189–193 (1991), the disclosures of which are hereby incorporated by reference. In accordance with the present invention, the ligase detection reaction can use 2 sets of complementary oligonucleotides. This is known as the ligase chain reaction which is described in the 3 immediately preceding references, which are hereby incorporated by reference. Alternatively, the ligase detection reaction can involve a single cycle which is known as the oligonucleotide ligation assay. See Landegren, et al., "A Ligase-Mediated Gene Detection Technique," *Science* 241:1077–80 (1988); Landegren, et al., "DNA Diagnostics—Molecular Techniques and Automation," *Science* 242:229–37 (1988); and U.S. Pat. No. 4,988,617 to Landegren, et al., which are hereby incorporated by reference

During ligase detection reaction phases, the denaturation treatment is carried out at a temperature of 80–105° C., while hybridization takes place at 50–85° C. Each cycle comprises a denaturation treatment and a thermal hybridization treatment which in total is from about one to five minutes long. Typically, the ligation detection reaction involves repeatedly denaturing and hybridizing for 2 to 50 cycles. The total time for the ligase detection reaction phase is 1 to 250 minutes.

The oligonucleotide probe sets or primers can be in the form of ribonucleotides, deoxynucleotides, modified ribonucleotides, modified deoxyribonucleotides, modified phosphate-sugar-backbone oligonucleotides, nucleotide analogs, and mixtures thereof.

In one variation, the oligonucleotides of the oligonucleotide probe sets each have a hybridization or melting temperature (i.e. T_m) of 66–70° C. These oligonucleotides are 20–28 nucleotides long.

The oligonucleotide probe sets or primers, as noted above, have a reporter label suitable for detection. Useful labels include chromophores, fluorescent moieties, enzymes, antigens, heavy metals, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moieties, and electrochemical detecting moieties.

The polymerase chain reaction process is fully described in H. Erlich, et. al., "Recent Advances in the Polymerase Chain Reaction," *Science* 252: 1643–50 (1991); M. Innis, et. al., *PCR Protocols: A Guide to Methods and Applications*, Academic Press: New York (1990); and R. Saiki, et. al., "Primer-directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase," *Science* 239:487–91 (1988), which are hereby incorporated by reference.

A particularly important aspect of the present invention is its capability to quantify the amount of target nucleotide

sequence in a sample. This can be achieved in a number of ways by establishing standards which can be internal (i.e. where the standard establishing material is amplified and detected with the sample) or external (i.e. where the standard establishing material is not amplified, and is detected with the sample).

In accordance with one quantification method, the signal generated by the reporter label, resulting from capture of ligation product sequences produced from the sample being analyzed, are detected. The strength of this signal is compared to a calibration curve produced from signals generated by capture of ligation product sequences in samples with known amounts of target nucleotide sequence. As a result, the amount of target nucleotide sequence in the sample being analyzed can be determined. This techniques involves use of an external standard.

Another quantification method, in accordance with the present invention, relates to an internal standard. Here, a known amount of one or more marker target nucleotide sequences is added to the sample. In addition, a plurality of marker-specific oligonucleotide probe sets are added along with the ligase, the previously-discussed oligonucleotide probe sets, and the sample to a mixture. The marker-specific oligonucleotide probe sets have (1) a first oligonucleotide probe with a target-specific portion complementary to the marker target nucleotide sequence, and (2) a second oligonucleotide probe with a target-specific portion complementary to the marker target nucleotide sequence and a detectable reporter label. The oligonucleotide probes in a particular marker-specific oligonucleotide set are suitable for ligation together when hybridized adjacent to one another on a corresponding marker target nucleotide sequence. However, there is a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the sample or added marker sequences. The presence of ligation product sequences is identified by detection of reporter labels. The amount of target nucleotide sequences in the sample is then determined by comparing the amount of ligation product sequence generated from known amounts of marker target nucleotide sequences with the amount of other ligation product sequences.

Another quantification method, in accordance with the present invention involves, analysis of a sample containing two or more of a plurality of target nucleotide sequences with a plurality of sequence differences. Here, ligation product sequences corresponding to the target nucleotide sequences are detected and distinguished by any of the previously-discussed techniques. The relative amounts of the target nucleotide sequences in the sample are then quantified by comparing the relative amounts of captured ligation product sequences generated. This provides a quantitative measure of the relative level of the target nucleotide sequences in the sample.

The preferred thermostable ligase is that derived from *Thermus aquaticus*. This enzyme can be isolated from that organism. M. Takahashi, et al., "Thermophillic DNA Ligase," *J. Biol. Chem.* 259:10041–47 (1984), which is hereby incorporated by reference. Alternatively, it can be prepared recombinantly. Procedures for such isolation as well as the recombinant production of *Thermus aquaticus* ligase (as well as *Thermus themophilus* ligase) are disclosed in WO 90/17239 to Barany, et. al., and F. Barany, et al., "Cloning, Overexpression and Nucleotide Sequence of a Thermostable DNA-Ligase Encoding Gene," *Gene* 109:1–11 (1991), which are hereby incorporated by reference. These references contain complete sequence information for this ligase as well as the encoding DNA. Other suitable ligases include *E. coli* ligase, T4 ligase, and Pyococcus ligase.

The ligation detection reaction mixture may include a carrier DNA, such as salmon sperm DNA.

The hybridization step in the ligase detection reaction, which is preferably a thermal hybridization treatment discriminates between nucleotide sequences based on a distinguishing nucleotide at the ligation junctions. The difference between the target nucleotide sequences can be, for example, a single nucleic acid base difference, a nucleic acid deletion, a nucleic acid insertion, or rearrangement. Such sequence differences involving more than one base can also be detected. Preferably, the oligonucleotide probe sets have substantially the same length so that they hybridize to target nucleotide sequences at substantially similar hybridization conditions. As a result, the process of the present invention is able to detect infectious diseases, genetic diseases, and cancer. It is also useful in environmental monitoring, forensics, and food science.

A wide variety of infectious diseases can be detected by the process of the present invention. Typically, these are caused by bacterial, viral, parasite, and fungal infectious agents. The resistance of various infectious agents to drugs can also be determined using the present invention.

Bacterial infectious agents which can be detected by the present invention include *Escherichia coli*, Salmonella, Shigella, Klebsiella, Pseudomzonas, *Listeria monocytogenes, Mycobacterium tuberculosis, Mycobacterium avium-intracellulare*, Yersinia, Francisella, Pasteurella, Brucella, Clostridia, *Bordetella pertussis*, Bacteroides, *Staphylococcus aureus, Streptococcus pneumonia*, B-Hemolytic strep., Corynebacteria, Legionella, Mycoplasma, Ureaplasma, Chlamydia, *Neisseria gonorrhea, Neisseria meningitides, Hemophilus influenza, Enterococcus faecalis, Proteus vulgaris, Proteus mirabilis, Helicobacter pylori, Treponema palladium, Borrelia burgdorferi, Borrelia recurrentis*, Rickettsial pathogens, Nocardia, and Acitnomycetes.

Fungal infectious agents which can be detected by the present invention include *Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Candida albicans, Aspergillus fumigautus*, Phycomycetes (Rhizopus), *Sporothrix schenckii*, Chromomycosis, and Maduromycosis.

Viral infectious agents which can be detected by the present invention include human immunodeficiency virus, human T-cell lymphocytotrophic virus, hepatitis viruses (e.g., Hepatitis B Virus and Hepatitis C Virus), Epstein-Barr Virus, cytomegalovirus, human papillomaviruses, orthomyxo viruses, paramyxo viruses, adenoviruses, corona viruses, rhabdo viruses, polio viruses, toga viruses, bunya viruses, arena viruses, rubella viruses, and reo viruses.

Parasitic agents which can be detected by the present invention include *Plasmodium falciparum, Plasmodium malaria, Plasmodium vivax, Plasmodium ovale, Onchoverva volvulus*, Leishmania, Trypanosoma spp., Schistosoma spp., *Entamoeba histolytica*, Cryptosporidum, Giardia spp., Trichimonas spp., *Balatidium coli, Wuchereria bancrofti*, Toxoplasma spp., *Enterobius vermicularis, Ascaris lumbricoides, Trichuris trichiura, Dracunculus medinesis*, trematodes, *Diphyllobothrium latum*, Taenia spp., *Pneumocystis carinii*, and *Necator americanis.*

The present invention is also useful for detection of drug resistance by infectious agents. For example, vancomycin-resistant *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae*, multi-drug resistant *Mycobacterium tuberculosis*, and AZT-resistant human immunodeficiency virus can all be identified with the present invention.

Genetic diseases can also be detected by the process of the present invention. This can be carried out by prenatal or post-natal screening for chromosomal and genetic aberrations or for genetic diseases. Examples of detectable genetic diseases include: 21 hydroxylase deficiency, cystic fibrosis, Fragile X Syndrome, Turner Syndrome, Duchenne Muscular Dystrophy, Down Syndrome or other trisomies, heart disease, single gene diseases, HLA typing, phenylketonuria, sickle cell anemia, Tay-Sachs Disease, thalassemia, Klinefelter Syndrome, Huntington Disease, autoimmune diseases, lipidosis, obesity defects, hemophilia, inborn errors of metabolism, and diabetes.

Cancers which can be detected by the process of the present invention generally involve oncogenes, tumor suppressor genes, or genes involved in DNA amplification, replication, recombination, or repair. Examples of these include: BRCA1 gene, p53 gene, APC gene, Her2/Neu amplification, Bcr/Ab1, K-ras gene, and human papillomavirus Types 16 and 18. Various aspects of the present invention can be used to identify amplifications, large deletions as well as point mutations and small deletions/insertions of the above genes in the following common human cancers: leukemia, colon cancer, breast cancer, lung cancer, prostate cancer, brain tumors, central nervous system tumors, bladder tumors, melanomas, liver cancer, osteosarcoma and other bone cancers, testicular and ovarian carcinomas, head and neck tumors, and cervical neoplasms.

In the area of environmental monitoring, the present invention can be used for detection, identification, and monitoring of pathogenic and indigenous microorganisms in natural and engineered ecosystems and microcosms such as in municipal waste water purification systems and water reservoirs or in polluted areas undergoing bioremediation. It is also possible to detect plasmids containing genes that can metabolize xenobiotics, to monitor specific target microorganisms in population dynamic studies, or either to detect, identify, or monitor genetically modified microorganisms in the environment and in industrial plants.

The present invention can also be used in a variety of forensic areas, including for human identification for military personnel and criminal investigation, paternity testing and family relation analysis, HLA compatibility typing, and screening blood, sperm, or transplantation organs for contamination.

In the food and feed industry, the present invention has a wide variety of applications. For example, it can be used for identification and characterization of production organisms such as yeast for production of beer, wine, cheese, yogurt, bread, etc. Another area of use is with regard to quality control and certification of products and processes (e.g., livestock, pasteurization, and meat processing) for contaminants. Other uses include the characterization of plants, bulbs, and seeds for breeding purposes, identification of the presence of plant-specific pathogens, and detection and identification of veterinary infections.

Desirably, the oligonucleotide probes are suitable for ligation together at a ligation junction when hybridized adjacent to one another on a corresponding target nucleotide sequence due to perfect complementarity at the ligation junction. However, when the oligonucleotide probes in the set are hybridized to any other nucleotide sequence present in the sample, there is a mismatch at a base at the ligation junction which interferes with ligation. Most preferably, the mismatch is at the base adjacent the 3' base at the ligation junction. Alternatively, the mismatch can be at the bases adjacent to bases at the ligation junction.

As noted supra, detection and quantification can be carried out using capillary or gel electrophoresis or on a solid support with an array capture oligonucleotides.

The use of capillary and gel electrophoresis for such purposes is well known. See e.g., Grossman, et. al., "High-density Multiplex Detection of Nucleic Acid Sequences: Oligonucleotide Ligation Assay and Sequence-coded Separation," *Nucl. Acids Res.* 22(21): 4527–34 (1994), which is hereby incorporated by reference.

The use of a solid support with an array of capture oligonucleotides is fully disclosed in pending provisional U.S. patent application Ser. No. 60/011,359, which is hereby incorporated by reference. When using such arrays, the oligonucleotide primers or probes used in the above-described coupled PCR and LDR phases, respectively, have an addressable array-specific portion. After the LDR or PCR phases are completed, the addressable array-specific portions for the products of such processes remain single stranded and are caused to hybridize to the capture oligonucleotides during a capture phase. C. Newton, et al., "The Production of PCR Products With 5' Single-Stranded Tails Using Primers That Incorporate Novel Phosphoramidite Intermediates," *Nucl. Acids Res.* 21(5):1155–62 (1993), which is hereby incorporated by reference.

During the capture phase of the process, the mixture is contacted with the solid support at a temperature of 45–90° C. and for a time period of up to 60 minutes. Hybridizations may be accelerated by adding cations, volume exclusion or chaotropic agents. When an array consists of dozens to hundreds of addresses, it is important that the correct ligation product sequences have an opportunity to hybridize to the appropriate address. This may be achieved by the thermal motion of oligonucleotides at the high temperatures used, by mechanical movement of the fluid in contact with the array surface, or by moving the oligonucleotides across the array by electric fields. After hybridization, the array is washed sequentially with a low stringency wash buffer and then a high stringency wash buffer.

It is important to select capture oligonucleotides and addressable nucleotide sequences which will hybridize in a stable fashion. This requires that the oligonucleotide sets and the capture oligonucleotides be configured so that the oligonucleotide sets hybridize to the target nucleotide sequences at a temperature less than that which the capture oligonucleotides hybridize to the addressable array-specific portions. Unless the oligonucleotides are designed in this fashion, false positive signals may result due to capture of adjacent unreacted oligonucleotides from the same oligonucleotide set which are hybridized to the target.

The capture oligonucleotides can be in the form of ribonucleotides, deoxyribonucleotides, modified ribonucleotides, modified deoxyribonucleotides, peptide nucleotide analogues, modified peptide nucleotide analogues, modified phosphate-sugar backbone oligonucleotides, nucleotide analogues, and mixtures thereof.

Where an array is utilized, the detection phase of the process involves scanning and identifying if LDR or PCR products have been produced and correlating the presence of such products to a presence or absence of the target nucleotide sequence in the test sample. Scanning can be carried out by scanning electron microscopy, confocal microscopy, charge-coupled device, scanning tunneling electron microscopy, infrared microscopy, atomic force microscopy, electrical conductance, and fluorescent or phosphor imaging. Correlating is carried out with a computer.

EXAMPLES

LDR/PCR Process

Example 1

Genomic DNA Preparation

Genomic DNA was prepared from the blood of two normal human volunteers, one male and one female, according to standard techniques. Briefly, approximately 12 ml of blood was obtained in EDTA-containing blood collection tubes. Red blood cells were lysed by mixing the blood samples with 4 volumes of lysis buffer (10 mM Tris pH 8.0, 10 mM EDTA). After 10 min on ice with occasional agitation, the suspensions were centrifuged and the supernatants were decanted. The white blood cell pellets were resuspended in 20 ml of lysis buffer, and the above process was repeated. Each cell pellet was then suspended in 15 ml of digestion buffer (50 mM Tris pH 8.0, 5 mM EDTA, 100 mM NaCl, 1% SDS) and 3 mg (0.2 mg/ml) of proteinase K was added. The cells were digested at 37° C. for 5 hours. The digests were extracted twice with equal volumes of phenol, then once with equal volumes of a 1:1 phenol:chloroform mixture and finally once with equal volumes of chloroform, each time centrifuging the mixture and removing the aqueous phase for the next extraction. After the final extraction and removing the aqueous phases, one tenth volume of 3 M sodium acetate, pH 6.5, was added. Two volumes of ice cold 100% EtOH were then added to each solution to precipitate the genomic DNAs, which were spooled out of solution on glass pipettes. The DNA precipitates were washed twice in 0.75 ml volumes of 70% EtOH, briefly centrifuging each time to allow removal of the supernatants. After removing the supernatants for the second time, the remaining EtOH was allowed to evaporate and the DNA was suspended in 0.5 ml of TE (10 mM Tri-HCl pH 8.0 containing 1 mM EDTA) solution. A fifth dilution of each DNA solution was also prepared in TE.

To determine the concentrations of the one fifth DNA solutions, 1, 2, and 4 μl aliquots of each were loaded on a 1% agarose gel with a known amount of HindIII digested lambda DNA as a control. The gel was run at 150 Volts for 2 hours with ethidium bromide in the electrophoresis buffer. After photographing the gel and comparing the intensities of the DNA bands, the one fifth dilutions were judged to have concentrations of approximately 100 ng/ml. DNA solutions extracted from various tumor cell lines were the generous gifts of other laboratories. The concentrations of these solutions were checked in a similar fashion and solutions of 100 ng/ml in TE were prepared.

To digest the genomic DNAs with Taq I, 25 μl of the 100 ng/μl solutions was mixed with 5 μl of 10×medium salt buffer (0.5 M NaCl, 0.1 M $MgCl_2$, 0.1 M Tris, pH 8.0), 20 μl of water-ME (i.e. water containing 6 mM ME (i.e., mercaptoethanol)), and 400 U of Taq I restriction endonuclease. The digests were covered with mineral oil and incubated at 65° C. for 1 hour. The reactions were stopped by adding 1.2 μl of 500 mM EDTA and heating the specimens to 85° C. for 10 min. Complete digestion of the DNAs was checked by electrophoresing aliquots on a 1% agarose gel.

Example 2

Oligonucleotide Preparation for LDR Probes and PCR Primers

All oligonucleotides were synthesized on a 394A DNA Synthesizer (Applied Biosystems Division of Perkin-Elmer Corp., Foster City, Calif.). Oligonucleotides labeled with 6-FAM were synthesized using the manufacturer's suggested modifications to the synthesis cycle (Applied Biosystems Inc., 1994) and were subsequently deprotected at 55° C. for 4 hr. LDR oligonucleotides were purified by ethanol precipitation after overnight deprotection at 55° C. The primer-specific portions of the oligonucleotides used for PCR amplification were purified by polyacrylamide gel electrophoresis on 10% acrylamide/7M urea gels. Oligonucleotides were visualized after electrophoresis by UV shadowing against a lightening screen and excised from the gel (Applied Biosystems Inc., 1992). They were then eluted overnight at 64° C. in TNE (i.e. Tris-sodium EDTA) buffer (100 mM Tris/HCl pH 8.0 containing 500 mM NaCl and 5 mM EDTA) and recovered from the eluate using Sep Pak cartridges (Millipore Corp, Milford, Mass.) following the manufacture's instructions.

Oligonucleotides were resuspended in 100 μl TE (i.e. 10 mM Tri-HCl pH 8.0 containing 1 mM EDTA). Typical concentrations of these original LDR probe solutions are about 1 μg/μl or approximately 74 pm/μl as determined by the following formula:

$$[\text{concentration } (\mu g/\mu l)\times 10^6]/[\text{length (nt)}\times 325]=\text{pm}/\mu l$$

The concentrations of the LDR probes are given in Table 1. The concentrations of oligonucleotides complementary to the oligonucleotide probes of the ligase detection reaction were higher. ZipALg1F was 3.75 μg/μl and ZipBLg2R was 2.01 μg/μl or 524 pm/μl and 281 pm/μl, respectively, as determined by the formula above.

TABLE 1

Primer	Length	μg/μl	pm/μl	Vol = 100 pm	1 = 200 pm
G6PDEx6-3L	48 nt	0.86	55.1	1.81 μl	3.63 μl
G6PDEx6-4R	48	0.65	41.7	2.4	4.8
ErbBExl-5L	48	0.95	60.9	1.64	3.28
ErbBExl-5R	48	1.4025	89.9	1.11	2.22
Int2Ex3-7L	50	1.6005	98.5	1.02	2.03
Int2Ex3-8R	46	1.306	87.4	1.14	2.29
p53Ex8-9L	2	1.036	61.3	1.63	3.26
p53Ex8-10R	44	1.164	81.4	1.23	2.46
SODEx3-11L	49	1.287	80.8	1.24	2.48
SODEx3-12R	47	1.2045	78.9	1.27	2.53

As a prerequisite for the LDR phase, the downstream LDR oligonucleotides probes were phosphorylated with T4 polynucleotide kinase. Aliquots of the 5 downstream oligonucleotides equivalent to 200 pm (see Table 1) were combined with 10 μl of 10×kinase buffer (500 mM Tris/HCl pH 8.0, 100 mM $MgCl_2$), 10 μl of 10 mM ATP, 20 U T4 kinase, and sufficient water-ME to give a final volume of 100 μl. Phosphorylation was carried out at 37° C. for 30 min followed by incubation for 10 min at 85° C. to inactivate the T4 enzyme. The resulting concentration of the kinased LDR probe solution was 2 pm/μl or 2000 fm/μl in each probe. The kinase reaction is summarized as follows:

4.8 μl	G6PDEx6-4R
2.2 μl	ErbBEx1-5R
2.3 μl	Int2Ex3-8R
2.5 μl	p53Ex8-10R
2.5 μl	SODEx3-12R
10 μl	10 × Kinase Buffer
10 μl	10 mMATP
65.7 μl	HOH + ME
100 μl	Total +
2 μl	= 20 units T4 Kinase

37° C. for 30 min.

Heat kill kinase, 85° C., 10 min.

Final concentration=2 pm/μl=2000 fm/μl

The solutions of the LDR and PCR oligonucleotides were adjusted to convenient concentrations. The kinased LDR probe solution was diluted fourfold in water to yield a concentration of 500 fm/μl. A solution of the upstream LDR probes was made by combining volumes of the probes equivalent to 200 pm (see Table 1) with sufficient water to give a final volume of 400 μl. This created a solution 500 fm/μl in each of the upstream LDR probes. Aliquots (20 μl) of the kinased and unkinased LDR probes were frozen for subsequent use. Standard solutions of the PCR primers (10 pm/μl) were prepared from their original solutions by combining 9.5 μl of ZipALg1F and 17.8 μl of ZipBLg2R with sufficient water to achieve a total volume of 500 μl. These solutions were frozen for use in the LDR/PCR process.

Unkinased probes were prepared according to the following:

200 pm ea Primer	
3.62 μl	G6PDEx6-3L
3.28 μl	ErbBEx1-5L
2.04 μl	Int2Ex3-7L
3.26 μl	p53Ex8-9L
2.48 μl	SODEx3-11L
385.32 μl	HOH
400 μl	Total Vol

Final concentration=0.5 pm/μl=500 fm/μl

TABLE 2

Gene Gene	Location	Sequences Probe (length in nt) Upstream	Downstream	Ligation Position
erb	17q12-q21	erbBEx1-5L	(48) erbBEx1-6R	(48) exon "1" P40
G6PD	Xq28	G6PDEx6-3L	(48) G6PDEx6-4R	(48) exon 6 W1145

TABLE 2-continued

Gene Gene	Location	Sequences Probe (length in nt) Upstream	Downstream	Ligation Position
Int2	11q13	Int2Ex3-7L	(50) Int2Ex3-8R	(46) exon 3 W135
p53	17p13.1	p53Ex8-9L	(52) p53Ex8-10R	(44) exon 8 P51
SOD	21q22.1	SODEx3-11L	(49) SODEx3-12R	(47) exon 3 P355

FIG. **24** shows the design of LDR oligonucleotide probes for quantification of gene amplifications and deletions in the LDR/PCR process. These oligonucleotide probes were designed to recognize exon 8 in the p53 tumor suppressor gene (on chromosome 17p), exon 3 of int-2 (on chromosome 11q), an internal exon in HER-2/neu (i.e. HER-2/neu/erbB oncogene) (on chromosome 17q), exon 3 in SOD (i.e. super oxide dimutase) (on chromosome 21q), and exon 6 in G6PD (i.e. glucose 6-phosphate dehydrogenase) (on chromosome Xq). Each pair of LDR oligonucleotide probes has the following features: (i) The left oligonucleotide probe contains from 5' to 3' an 18 base sequence identical to the fluorescently labeled secondary oligonucleotide primer (black bar), an "adjustment sequence" (white bar), and a target-specific sequence of from 22 to 28 bases with a T_m of 75° C. (patterned bar); (ii) The right oligonucleotide probe contains from 5' to 3' a target-specific sequence of 20–25 bases with a T_m of 75° C. (patterned bar), a single HaeIII or HinP1I restriction site at slightly different positions within the target-specific sequence, and an "adjustment sequence" (white bars). The two oligonucleotide probes are designed such that their combined length is exactly 96 bases, with 50 G+C bases and 46 A+T bases. The position of each unique restriction site generates a product which differs by at least 2 bases from the other products. Each oligonucleotide probe set has an exon-specific region chosen to ligate the junction sequence of (A, T)C↓C(A, T). This junction sequence corresponds to either a proline residue (codon CCN) or the complementary sequence of a tryptophan residue (TGG). These sequences were chosen to minimize differences in ligation rates and the chance of a polymorphism at the ligation junction.

LDR Probe Sequences

G6PDEx6-3L 5'CAC GCT ATC CCG TTA GAC ATT GTC AAG CAG GCG ATG TTG TCC CGG TTC 3'
(SEQ. ID. NO. 1)
G6PDEx6-4R 5'CAG ATG GGG CCG AAG ATC CTG TTA TTG ATA CAT AGT GCG GTA GTT GGC 3'
(SEQ. ID. NO. 2)
erbBEx1-5L 5'CAC GCT ATC CCG TTA GAC ATC GCC CTG ATG GGG AGA ATG TGA AAA TTC 3'
(SEQ. ID. NO. 3)
erbBEx1-6R 5'CAG TGG CCA TCA AAG TGT TGA GGG AGC GTA CAT AGT GCG GTA GTT GGC 3'
(SEQ. ID. NO. 4)
Int2Ex3-7L 5'CAC GCT ATC CCG TTA GAC ATT CAT AAC CCT TGC CGT TCA CAG ACA CGT AC 3'
(SEQ. ID. NO. 5)
Int2Ex3-8R 5'CAC AGT CTC TCG GCG CTG GGC AAT AAT ACA TAG TGC GGT AGT TGG C 3'
(SEQ. ID. NO. 6)
p53Ex8-9L 5'CAC GCT ATC CCG TTA GAC ATC TTA GTA ATT GAG GTG CGT GTT TGT GCC TGT C 3'
(SEQ. ID. NO. 7)
p53Ex9-10R 5'CTG GGA GAG ACC GGC GCA CAT TAC TAC ATA GTG CGG TAG TTG GC 3'
(SEQ. ID. NO. 8)
SODEx-3-11L 5'CAC GCT ATC CCT TTA GAC ATC TGT ACC AGT GCA GGT CCT CAC TTT AAT C 3'
(SEQ. ID. NO. 9)
SODEx-3-42R 5'CTC TAT CCA GAA AAC ACG GTG GGC CGC TAC ATA GTG CGG TAG TTG GC 3'
(SEQ. ID. NO. 10)

-continued

PCR Primers:

ZipALg1F 5' Fam-GGA GCA CGC TAT CCC GTT AGA C 3' (Tm = 71° C.)
(SEQ. ID. NO. 11)
ZipBLg2R 5' CGC TGC CAA CTA CCG CAC TAT G 3' (Tm = 72° C.)
(SEQ. ID. NO. 12)
(Underlined sequences are common between LDR probes and ZIPALg1F or the complement of ZipBLg2R.)

Example 3

Buffers and Reagents

A. LDR Buffers/Reagents—the following LDR buffers and reagents were selected:

10×ST ligase buffer (0.2 M Tris pH 8.5, 0.1 M $MgCl_2$) [This was also tested with Tris at pH 7.6.]

10×TT ligase buffer (0.2 M Tris pH 7.6, 0.5 M KCl, 0.1 M $MgCl_2$, 5 mM EDTA)

NAD (10 mM)

DTT (200 mM)

LDR primer solution containing one tenth concentration of each of the LDR primer mixtures (50 fm of each LDR primer per μl)

Tth DNA Ligase (625 U/μl)

B. PCR Buffers/Reagents—the following PCR buffers and reagents were selected:

10×Stoffel buffer (0.1 M KCl, 0.1M Tris-HCl pH 8.3 Perkin Elmer)

dNTP solution (100 mM total, 25 mM of each dNTP Perkin Elmer), diluted 5 fold in dHOH to a final concentration of 5 mM of each dNTP

ZipALg1F (10 pm/μl)

ZipBLg2R (10 pm/μl)

Example 4

LDR/PCR Process

Four LDR/PCR processes were performed for each DNA to be tested with reaction tubes PCR amplified to 22, 24, 26, and 30 cycles to assure that one reaction would be halted in the exponential phase. Each LDR reaction (20 μl) was thermal cycled and then a PCR mix (30 μl) containing primers with a portion complementary to the primer-specific portion of the LDR probes was added to each specimen to allow exponential amplification. To minimize differences between reaction tubes, master mixes of LDR and PCR reagents were made.

A master mix of LDR reagents was constructed with a volume sufficient for all reaction tubes. Proportions and volumes for a single reaction were as follows:

Reagent	Volume	
10X ST Ligase Buffer	2 μl	
NAD (10 mM)	2 μl	
DTT (200 mM)	1 μl	
dHOH	5 μl	
Total	10 μl	
Tth DNA Ligase	0.2 μl	(= 125 U)

Mixes of target DNA and LDR probes for each reaction tube were constructed with the following proportions:

Reagent	Volume	
DNA (TaqI digested)	1 μl	(= 50 ng)
LDR Probe Mix	4 μl	(200 fm each primer)
dHOH	5 μl	
Total	10 μl	

For each reaction, 10 μl was placed in a thin-walled PCR tube, rapidly mixed with 10 μl of LDR reagent mix (including ligase), overlayed with mineral oil, and placed in a Perkin Elmer 9600 thermal cycler.

LDR was initiated by holding at 96° C. for 2 minutes to denature the DNA followed by 10 cycles of 94° C. for 30 seconds and 65° C. for 4 minutes. PCR reagent mixes for each reaction tube were constructed with the following proportions:

Reagent	Volume	
10X Stoffel buffer	5 μl	
dNTP solution	8 μl	(= 0.8 mM each dNTP in final reaction)
ZipALg1F (10 pm/μl)	2.5 μl	(= 25 pm per reaction)
ZipBLg2R (10 pm/μl)	2.5 μl	(= 25 pm per reaction)
dHOH	12 μl	
Total	30 μl	
Stoffel Fragment	0.25 μl	(= 2.5 U)

At the completion of the LDR reaction, the tubes were held at 94° C., while 30 ml of PCR reagent mix (including Stoffel fragment) were added to each tube. PCR amplification was accomplished by thermal cycling at 94° C. for 15 seconds followed by 60° C. for 50 seconds. At 22, 24, 26, and 30 cycles, respectively, one of four identical reaction tubes of each DNA specimen was removed and quenched in a slurry of dry ice and ETOH.

Example 5

Agarose Gel Evaluation

Ten microliter aliquots of the 26 and 30 cycle reaction specimens were evaluated on a 2% agarose gel. Ethidium bromide staining revealed bands of the expected size (104 bp).

Example 6

Digestion of Products, Preparation of Dilutions, and Loading on GeneScanner

To separate the gene-specific LDR/PCR products, 10 μl aliquots of the 22, 24, and 26 cycle reactions were digested by adding 10 μl of a solution containing 5 U each of HaeIII and HinP1I restriction enzymes (both from New England BioLabs), 2 μl of 10×restriction enzyme buffer number 2 (New England BioLabs), and 8 μl of dHOH (i.e. distilled

water). The digests were incubated at 37° C. for one hour and then stopped by the addition of 1 μl of 0.5 M EDTA, pH 8.0. The restriction digests were a one half dilution of the original LDR/PCR products. A 10 fold dilution of each sample was also prepared by adding 5 μl of each restriction digest to 20 μl of TE buffer.

Before loading samples on the ABI 373A DNA Sequencer (Applied Biosystems) a 1:5 mixture of 50 mM EDTA pH 8.0 containing 2% blue dextran and de-ionized formamide was made. To 5 μl of the EDTA-Blue Dextran solution, 5 μl of digested LDR/PCR product dilution and 1 μl of GENESCAN 1000 ROX marker (Applied Biosystems) were added. These solutions were heated to 85° C. for 10 minutes and snap chilled on ice, before 5.5 μl were loaded on the denaturing gel.

Samples were analyzed in an Applied Biosystems 373A DNA sequencer on a 0.4 mm thick, 10% polyacrylamide/7M urea gel with a well-to-read distance of 12 cm. The gel matrix was buffered with 1.2×TBE (106 mM Tris-borate and 2.4 mM EDTA pH 8.3) and the electrophoresis chamber buffer contained 0.6×TBE (53.4 mM Tris-borate and 1.2 mM EDTA pH 8.3). The gel was pre-run prior to sample loading at 1600 V for 30 minutes with the electrode polarity reversed (anode in the chamber with sample wells at the top of the gel). After loading, the gene-specific LDR/PCR products were electrophoresed at 1200 V and the primary data was captured using the ABI 672 Data Collection Software V1.1 (Applied Biosystems).

Using the ABI 672 GeneScan Analysis Software V1.2.2 (Applied Biosystems), the resulting data were displayed as electropherograms, with peak heights and peak areas calculated.

In the normal female, the ErbB2 peak is lower, and the p53 peak is slightly lower than the remaining 3 peaks. See FIGS. **25**A–D. In different experiments, it was observed that the ErbB2 peak is always lower; the G6PD, Int-2, p53, and SOD peak areas would vary somewhat, but all 5 peaks would retain the same relative profile from one sample to the next for a given experiment. When comparing male with female DNA, the G6PD peak was about half the area of other peaks, consistent with a single X-chromosome in males, while the other peaks were essentially the same. The ErB2 peak for the NM10 Breast Cancer cell line is slightly elevated, while that in cell line SKBR3 is several fold greater than the normal female control, reflecting the known ErbB-2 gene amplification in these two cell lines. In addition, cell line NM10 appears to have undergone LOH (i.e. a loss of heterozygosity) of p53, while cell line SKBR3 appears to have undergone LOH of G6PD and p53. Some of the cells in cell line SKBR3 may have lost both copies of the p53 gene. Repeating these amplifications in the absence of the ErbB-2 primers was used to confirm the presence of these additional gene deletions (see below).

These results can be quantified by comparing the ratio of peak areas in each peak to a standard (the SOD peak area) for that experiment. The raw data and ratio of peak areas are given below:

TABLE 3

Raw Peak Area Data

	Genes				
	ErB	G6PD	Int2	p53	SOD
Male	9954	21525	45688	36346	62506
Female	8340	39309	39344	30270	54665

TABLE 3-continued

Raw Peak Area Data

	Genes				
	ErB	G6PD	Int2	p53	SOD
NM10	20096	55483	67083	17364	84339
SKBR3	106650	19120	50103	2888	48119

TABLE 4

Ratio of Peak Areas to SOD Peak Area

	ErbB/SOD	G6PD/SOD	Int2/SOD	p53/SOD
Male	0.16	0.34	0.73	0.58
Female	0.15	0.72	0.72	0.55
NM10	0.24	0.66	0.80	0.21
SKBR3	2.22	0.40	1.04	0.06

Although the ratios differ for each gene, (due to different efficiencies of LDR/PCR for each gene,) the ratios are generally consistent between the male and female sample, except for the G6PD/SOD ratio. The G6PD for the female is about twice the value as the male, accurately reflecting the presence of two and one X chromosome, respectively. One can quantify the amount of ErbB2 amplification by comparing the ratio of peak area ratios between normal DNA and cancer cell lines.

TABLE 5

Ratio of Peak Areas Ratios

	ErbB/2	G6PD	Int2	p53
Female/Male	0.96	2.09	0.98	0.95
NM10/Male	1.50	1.91	1.09	0.35
SKBR3/Male	13.92	1.15	1.42	0.10

From these ratios, it can be determined that the normal male and female have the same number of genes on chromosomes 17q (ErbB), 17p (p53), and 11q (Int 2), but that the female has twice as many G6PD genes, or X chromosomes. Likewise, cell line NM10 showed slight amplification of the ErbB-2 gene, and LOH at p53, while cell line SKBR3 shows significant amplification of the ErbB-2 gene, LOH at G6PD and p53. To confirm additional gene amplifications and deletions, primer pairs causing massive amplifications may be removed from the LDR/PCR reaction (see below).

In the normal female, the ErbB2 peak is lower than the remaining 4 peaks. In different experiments, it was observed that the G6PD, Int-2, p53, and SOD peak areas would vary somewhat, but would retain the same relative profile from one sample to the next. See FIGS. **26**A–C. The ErbB2 peak was consistently lower, and slight shoulders were observed on the G6PD and SOD peaks, for unknown reasons. The ErbB-2 peak in both cell line samples is several fold greater than the normal female control, reflecting the known ErbB-2 gene amplification in these two cell lines. In addition, the ZR-75-30 line appears to show LOH of p53, while the SKGT-2 cell line appears to have a slight amplification of the Int-2 region. By repeating these LDR/PCR experiments in the absence of the ErbB-2 primers, it was demonstrated that these results are not artifacts of the massive levels of ErbB-2 amplification. See FIGS. **27**A–C. Both gene amplifications and deletions for multiple genes using the LDR/PCR format have been demonstrated. See FIGS. **26**A–C and **27**A–C.

Again, these results can be quantified by comparing the ratio of peak areas in each peak to a standard (the SOD peak area) for that experiment. The raw data and ratio of peak areas are given below:

TABLE 6

Raw Peak Area Data

	Genes				
	ErbB	G6PD	Int2	p53	SOD
Female; 4 Primer Sets	NA	9577	8581	9139	8128
ZR7530; 4 Primer Sets	NA	8452	7904	4168	7996
SKGT2; 4 Primer Sets	NA	15915	28614	13116	12478
Female; 5 Primer Sets	3955	9436	8066	9304	8848
ZR7530; 5 Primer Sets	66748	11105	8812	4163	9303
SKGT2; 5 Primer Sets	263254	21877	31887	13630	13480

TABLE 7

Ratio of Peak Areas to SOD Peak Area

	ErbB/SOD	G6PD/SOD	Int2/SOD	p53/SOD
Female; 4 Primer Sets	NA	1.18	1.06	1.12
ZR7530; 4 Primer Sets	NA	1.06	0.99	0.52
SKGT2; 4 Primer Sets	NA	1.28	2.29	1.05
Female; 5 Primer Sets	0.45	1.97	0.91	1.05
ZR7530; 5 Primer Sets	7.17	1.19	0.95	0.45
SKGT2; 5 Primer Sets	19.53	1.62	2.37	1.01

The ratios are remarkably consistent between the four primer set and the five primer set experiments. The only exception is the G6PD peak for the SKGT2 cell line, where the huge peak for ErbB-2 may have added to the G6PD peak.

One can quantify the amount of ErbB2 and Int-2 amplification as well as p53 deletion by comparing the ratio of peak area ratios between normal DNA and cancer cell lines, as shown in Table 8. In addition, the ratios from using 4 sets of primers can be compared with 5 sets of primers to ascertain the internal consistency of this technique.

TABLE 8

Ratio of Peak Area Ratios

	ErbB	G6PD	Int2	p53
Female; 4/5	NA	1.10	1.16	1.07
ZR7530; 4/5	NA	0.89	1.04	1.16
SKGT2; 4/5	NA	0.79	0.97	1.04
ZR7530/Female; 4/4	NA	0.90	0.94	0.46
ZR7530; Female; 5/5	16.05	1.12	1.04	0.43
SKGT2/Female; 4/4	NA	1.08	2.17	0.93
SKGT2; Female; 5/5	43.69	1.52	2.59	0.96

The values for the top half of Table 8 should all be close to 1.0 if the LDR/PCR technique is internally consistent, when using 4 or 5 primers. All values are very close to 1.0. Again, the value for G6PD for SKGT2 is a bit low for the reasons mentioned.

The values on the bottom half of Table 8 show the extent of ErbB-2 amplification. The numbers are quite consistent for the 4 primer and 5 primer amplifications (with the exception of SKGT2-G6PD noted above). The ZR7530 cell line demonstrates a clear LOH for p53, while the SKGT2 cell line shows amplification of the Int-2 region, and both p53 genes present

Primary PCR/Secondary PCR/LDR Process

Example 7

Oligonucleotide Synthesis

Oligonucleotides were assembled by standard phosphoramidite chemistry on an Expedite DNA synthesizer (Perseptive Biosystems, Framingham, Mass.). Oligonucleotides 5'-end labeled with 6-FAM, TET, and HEX were synthesized using the appropriate dye phosphoramidites (Perkin Elmer-Applied Biosystems) and purified with Oligonucleotide Purification Cartridges (Perkin Elmer-Applied Biosystems) following the manufacturer's protocol (Applied Biosystems Division-Perkin Elmer Corp., "Synthesis and Purification of Fluorescently Labeled Oligonucleotides Using Dye Phosphoramidites," *User Bulletin*, number 78, Applied Biosystems Division, Foster City, Calif., (1994)), which is hereby incorporated by reference. All oligonucleotides were checked for purity on an Applied Biosystems 270-HT capillary electrophoresis instrument using a mPAGE-3 column (J&W Scientific, Folsom, Calif.). Only oligonucleotides that were greater than 95% pure were used for the experiments. Oligonucleotides were resuspended in 250 ml TE (10 mM Tris/HCl and 5 mM EDTA pH 8.0). Typical concentrations were 300–500 mM for crude stock solutions and 100–200 mM for OPC (i.e. Oligonucleotide Purification Columns available from Applied Biosystems) purified stock solutions. For PCR and LDR, oligonucleotides were diluted to working solutions of 10 mM (10 pmoles/ml) or 5 mM (5 pmoles/ml).

Example 8

Phosphorylation of LDR Oligonucleotides

The 12 LDR common oligonucleotides were phosphorylated at the 5' end to permit ligation to the fluorescent labeled oligonucleotides. The oligonucleotides are shown below in Table 9.

TABLE 9

LDR Oligonucleotide Sequences

Locus	Allele-Specific Oligonucleotide (5'->3')	Common Oligonucleotide (5'->3')
1	FAM-AGCTTCAATGATGAGAACCTGC (SEQ. ID. NO. 13) TET-AGCTTCAATGATGAGAACCTGT (SEQ. ID. NO. 15)	P-GCATAGTGGTGGCTGACCTGTTCATAT (SEQ. ID. NO. 14)

TABLE 9-continued

LDR Oligonucleotide Sequences

Locus	Allele-Specific Oligonucleotide (5'->3')	Common Oligonucleotide (5'->3')
2	FAM-CTCCATGGGCCCAGCC (SEQ. ID. NO. 16)	P-AGCACTGGTGCCCTGTGAG (SEQ. ID. NO. 17)
	TET-CTCCATGGGCCCAGCT (SEQ. ID. NO. 18)	
3	FAM-GGGGACAGCCATGCACTGA (SEQ. ID. NO. 19)	P-GCCTCTGGTAGCCTTTCAACCATA (SEQ. ID. NO. 20)
	TET-GGGGACAGCCATGCACTGC (SEQ. ID. NO. 21)	
4	FAMTTAGAAATCATCAAGCCTAGGTCAT (SEQ. ID. NO. 22)	P-CACCTTTTAGCTTCCTGAGCAATGAT (SEQ. ID. NO. 23)
	TET-TTAGAAATCATCAAGCCTAGGTCAG (SEQ. ID. NO. 24)	
5	HEX-GGTTGTATTTGTCACCATATTAATTA (SEQ. ID. NO. 25)	P-ATTTTTCTCTATTGTTTTCATCTTTCAGGA (SEQ. ID. NO. 26)
	HEX-ATGGTTGTATTTGTCACCATATTAATTG (SEQ. ID. NO. 27)	
6	FAM-GGGCCAAGAAGGTATCTACCA (SEQ. ID. NO. 28)	P-ATAGTGTCTATTAGGCATTTGAAAATGTGTAT (SEQ. ID. NO. 29)
	TET-GGGCCAAGAAGGTATCTACCG (SEQ. ID. NO. 30)	
7	FAM-ACACAGCAGCTTACTCCAGAGG (SEQ. ID. NO. 31)	P-TCAAGTCCAAGGCCATTGGCTTATA (SEQ. ID. NO. 32)
	TET-ACACAGCAGCTTACTCCAGAGA (SEQ. ID. NO. 33)	
8	FAM-CCAGCAAAGAGAAAAGAAGGG (SEQ. ID. NO. 34)	P-CCCCAGAAATCACAGGTGGGCTAT (SEQ. ID. NO. 35)
	TET-CCAGCAAAGAGAAAAGAAGGA (SEQ. ID. NO. 36)	
9	FAM-ATGATATTAGAGCTCACTCATGTCCA (SEQ. ID. NO. 37)	P-TCAGTTTGGAAAAAGACAAAGAATTCTTT (SEQ. ID. NO. 38)
	TET-ATGATATTAGAGCTCACTCATGTCCG (SEQ. ID. NO. 39)	
10	HEX-TGCTGTCTTCCAGGAATCTGTT (SEQ. ID. NO. 40)	P-CAACTCTCTCGAAGCCATGTTCACAA (SEQ. ID. NO. 41)
	HEX-ATTGCTGTCTTCCAGGAATCTGTG (SEQ. ID. NO. 42)	
11	HEX-GGACATAGTGACCGTGCAGGTC (SEQ. ID. NO. 43)	P-CTTCCCCAGTGTGAGTGCCGTA (SEQ. ID. NO. 44)
	HEX-ATGGACATAGTGACCGTGCAGGTT (SEQ. ID. NO. 45)	
12	HEX-CTATGACACCGTCATCAGCAGG (SEQ. ID. NO. 46)	P-GACATCCAGGCCCCCGAC (SEQ. ID. NO. 47)
	HEX-TACTATGACACCGTCATCAGCAGA (SEQ. ID. NO. 48)	

The allele-specific oligonucleotides are 5' end labeled with either FAM, TET, or HEX. All the common oligonucleotides are phosphorylated at the 5' end. Underline denotes tails that are not complementary to the target sequence. LDR primer sets were designed in two ways: (i) allele-specific primers were of the same length but contained either FAM or TET label; or (ii) theallele-specific primers were both labeled with HEX but differed in length by two bases.

This was accomplished either during the synthesis with Phosphate-ON (Clontech Laboratories, Palo Alto, Calif.) according to the manufacturer's instructions or post-synthesis, using T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, Ind.). In the latter, a common oligomer was diluted into 50 μl of kinase buffer (50 mM Tris/HCl, 10 mM $MgCl_2$, and 1 mM ATP) to a final concentration of 1 mM (500 pmol in 50 μl). Ten units of T4 kinase was added, and the reaction was incubated at 37° C. for 30 min. The T4 kinase was inactivated by heating at 95° C. for 10 min.

The kinase reaction was carried out as follows:

10 μl	50 mM common oligo
5 μl	10x kinase buffer
5 μl	10 mM ATP
30 μl	H2O
50 μl	Total +
1 μl	= T4 Kinase (10 units)

37° C. for 30 min.

95° C. for 10 min.

Final concentration=10 mM

Example 9

Multiplex PCR Amplification

Twelve gene regions (2–13) were chosen for simultaneous PCR amplification based on information available in the Human Genome Database, as shown below in Table 10.

Each region was well characterized and harbored a single-base variation with only two known alleles. PCR amplifications were performed using genomic DNA isolated from whole blood using the Purgene DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, Mich.) according to the manufacturer's instructions.

A volume of 25 μl of PCR buffer (10 mM Tris/HCl pH 8.3, 10 mM KCl, 4 mM $MgCl_2$, 0.4 mM each dNTP), 10–100 ng of genomic target DNA, PCR hybrid primer pairs 1–12 (2 pmol of each primer), and 1.3 units of AmpliTaq DNA

TABLE 10

List of Polymorphic Sites Analyzed

Site Number	Locus Symbol	Locus Name	Chr. Location	Nucleotide Position	Variation	Het.	Ref.
1	CYP2D6	cytochrome P450 IID6	22q13.1	4469 (M33388)	C, T	.38	2
2	AT3	antithrombin III	1q23–q25.1	7987 (X68793)	C, T	.46	3
3	C6	complement component C6	5p14–p12	185 (X72179)	A, C	.47	4
4	IL1A	interleukin 1 alpha	2q13	6282 (X03833)	T, G	.34	5
5	NF1	neurofibromatosis	17q11.2	63683 (L05367)	A, G	.47	6
6	ALDOB	aldolase B	9q22.3–q31	1087 (M15656)	A, G	.50	7
7	A2M	alpha 2 macroglobulin	12p13.3–p12.3	153 (X68731)	G, A	.42	8
8	IGF2	insulin growth factor	11p15.5	820 (X07868)	G, A	.46	9
9	PROS1	protein S alpha	3p11–cen	183 (M36564)	A, G	.50	10
10	LIPC	triglyceride lipase	15q21–q23	113 (M29189)	T, G	.49	11
11	CD18	integrin B-2 subunit	21q22.3	109 (X64081)	C, T	.50	12
12	LDLR	low density lipoprotein receptor	19pl3.2	70 (L00344)	G, A	.50	13

The site numbers are specific single point variations located within the respective genes. All variations indicated are defined on the sense strand. Genbank accession numbers are indicated in parentheses. Chr., chromosome; Het., heterozygosity.

References in Table 10

2 M. Armstrong, et al., "A Polymorphic Cfo I Site In Exon 6 of the Human Cytochrome P450 CYPD6 Gene Detected by the Polymerase Chain Reaction," Human Genetics 91:616–17 (1993), which is hereby incorporated by reference.

3 S. C. Bock, et al., "Antithrombin III Utah: Proline-407 to Leucine Mutation in a Highly Conserved Region Near the Inhibitor Reactive Site," Biochemistry 28:3628 (1991), which is hereby incorporated by reference.

4 G. Dewald, et al., "Polymorphism of Human Complement Component C6: An Amino Acid Substitution (glu/ala) Within the Second Thrombospondin Repeat Differentiates Between the Two Common Allotypes C6 A and C6 B," Biochem, Biophys. Res. Commun. 194:458–64 (1993), which is hereby incorporated by reference.

5 P. A. Velden, et al., "Amino Acid Dimorphism in IL1A is Detectable by PCR Amplification," Hum. Mol. Genet. 2:1753 (1993), which is hereby incorporated by reference.

6 R. M. Cawthon, et al., "Identification and Characterization of Transcripts From Theneurofibromatosis 1 Region: The Sequence and Genomic Structure of EV12 and Mapping of Other Transcripts," Genomics 7:555–65 (1990), which is hereby incorporated by reference.

7 C. C. Brooks, et al., "Association of the Widespread A149P Hereditary Fructose Intolerance Mutation With Newly Identified Sequence Polymorphisms in the Aldolase B Gene," Am. J. Human Genetics 52:835–40 (1993), which is hereby incorporated by reference.

8 W. Poller, et al., "Sequence Polymorphism in the Human Alpha-2-Macroglobulin (A2M) Gene," Nucleic Acids Res. 19:198 (1991), which is hereby incorporated by reference.

9 T. Gloudemans, "An Ava II Restriction Fragment Length Polymorphism in the Insulin-Like Growth Factor II Gene and the Occurrence of Smooth Muscle Tumors," Cancer Res. 53:5754–58 (1993), which is hereby incorporated by reference.

10 C. M. Diepstraten, et al., "A CCA/CCG Neutral Dimorphism in the Codon for Pro 626 of the Human Protein S Gene PSa (PROS1)," Nucleic Acids Res. 19:5091 (1991), which is hereby incorporated by reference.

11 M. Reina, et al., "SSCP Polymorphism in the Human Hepatic Triglyceride Lipase (LIPC) Gene," Hum. Mol. Genet. 1:453 (1992), which is hereby incorporated by reference.

12 S. Mastuura, et al., "Investigation of the Polymorphic AvaII Site by a PCR-based Assay at the Human CD18 Gene Locus," Human Genetics 93:721 (1994), which is hereby incorporated by reference.

13 L. Warnich, et al., "Detection of a Frequent Polymorphism in Exon 10 of the Low-Density Lipoprotein Receptor Gene," Human Genetics 89:362 (1992), which is hereby incorporated by reference.

polymerase Stoffel fragment (Applied Biosystems) was placed in a thin-walled MicroAmp reaction tube (Applied Biosystems). Each hybrid primer consisted of a gene-specific 3' region (16–29 bases) and a 5' region (22 bases) corresponding to one of two sets of universal (i.e. the portions which are primer-specific) primers (See FIG. **4**, where F1=Tet, F2=Fam, and F3=Het). These primers are shown in Table 11.

TABLE 11

Primary PCR Primer Sequences

Number or Name	Primer (5'→3')	
1	F GGAGCACGCTATCCCGTTAGAC**A**GCCAAGGGGAACCCTGAGAG	(SEQ. ID. NO. 49)
	R CGCTGCCAACTACCGCACTATG**AT**CGTGGTCGAGGTGGTCACCATC	(SEQ. ID. NO. 50)
2	F CCTCGTTGCGAGGCGTATTCTG**TA**TTTCCTCTTCTGTAAAAGGGAAGTTTGT	(SEQ. ID. NO. 51)
	R GCGACCTGACTTGCCGAAGAAC**AT**GTCCCATCTCCTCTACCTGATAC	(SEQ. ID. NO. 52)
3	F GGAGCACGCTATCCCGTTAGAC**TA**AAGATCTGTCTTGCGTCCCAGTCA	(SEQ. ID. NO. 53)
	R CGCTGCCAACTACCGCACTATG**TA**TCAATTTTGCAGAGCTTAGATGGAATG	(SEQ. ID. NO. 54)
4	F CCTCGTTGCGAGGCGTATTCTG**TA**GCACTTGTGATCATGGTTTTAGAAATC	(SEQ. ID. NO. 55)
	R GCGACCTGACTTGCCGAAGAAC**TA**TCGTATTTGATGATCCTCATAAAGTTG	(SEQ. ID. NO. 56)
5	F GGAGCACGCTATCCCGTTAGAC**AT**CAGCCACTTGGAAGGAGCAAAC	(SEQ. ID. NO. 57)
	R CGCTGCCAACTACCGCACTATG**AT**GGACCATGGCTGAGTCTCCTTTAG	(SEQ. ID. NO. 58)
6	F CCTCGTTGCGAGGCGTATTCTG**AA**CCAACACGGAGAAGCATTGTTTTC	(SEQ. ID. NO. 59)
	R GCGACCTGACTTGCCGAAGAAC**TA**TTAGCCTCAATCCTCATACTGACCTCTAC	(SEQ. ID. NO. 60)
7	F GGAGCACGCTATCCCGTTAGAC**AT**CTCCTAACATCTATGTACTGGATTATCTAAATG	(SEQ. ID. NO.61)
	R CGCTGCCAACTACCGCACTATG**AT**CTTACTCAAGTAATCACTCACCAGTGTTG	(SEQ. ID. NO. 62)
8	F CCTCGTTGCGAGGCGTATTCTG**AA**TGAGTCAAATTGGCCTGGACTTG	(SEQ. ID. NO. 63)
	R GCGACCTGACTTGCCGAAGAAC**TT**AATTCCCGTGAGAAGGGAGATG	(SEQ. ID. NO. 64)
9	F CCTCGTTGCGAGGCGTATTCTG**AA**GGATCTGGATGAAGCCATTTCTAAAC	(SEQ. ID. NO. 65)
	R GCGACCTGACTTGCCGAAGAAC**TT**GGAAAAGGTATTATAAGCAGAGAAAAGATG	(SEQ. ID. NO. 66)
10	F GGAGCACGCTATCCCGTTAGAC**A**GGACCGCAAAAGGCTTTCATC	(SEQ. ID. NO. 67)
	R CGCTGCCAACTACCGCACTATG**TA**GCACCCAGGCTGTACCCAATTAG	(SEQ. ID. NO. 68)
11	F CCTCGTTGCGAGGCGTATTCTG**AT**CGGGCGCTGGGCTTCAC	(SEQ. ID. NO. 69)
	R GCGACCTGACTTGCCGAAGAAC**AT**CAGATGCCGCACTCCAAGAAG	(SEQ. ID. NO. 70)
12	F GGAGCACGCTATCCCGTTAGAC**ATA**AGAGCCCACGGCGTCTCTTC	(SEQ. ID. NO. 71)
	R CGCTGCCAACTACCGCACTATGTAAGAGACAGTGCCCAGGACAGAGTC	(SEQ. ID. NO. 72)
ZipALg1	FGGAGCACGCTATCCCGTTAGAC	(SEQ. ID. NO. 73)
ZipBLg2	RCGCTGCCAACTACCGCACATG	(SEQ. ID. NO. 74)
ZipCLg3	F CCTCGTGCGAGGCGTATTCTG	(SEQ. ID. NO. 75)
ZipDLg4	R GCGACCTGACTTGCCGAAGAAC	(SEQ. ID. NO. 76)

Solid underline denotes the ZipALg1 and ZipBLg2 sequences. Dotted underline denotes the ZipCLg1 and ZtpDLg2 sequences. Linker sequences are indicated in bold. F=forward, R=reverse.

Forward and reverse hybrid primers for loci 1, 3, 5, 7, 10, and 12 contained 5' end regions identical to universal primers ALg1 and BLg2 respectively. Forward and reverse primers to loci 2, 4, 6, 8, 9, and 11 contained 5' end regions identical to universal primers CLg3 and DLg4, respectively. The rationale for using a low concentration of the hybrid primers in the PCR phase was to deplete the hybrid primers during the reaction. This would theoretically allow products with low amplification efficiencies to "catch up with" those that had high amplification efficiencies. Amplification was attained by thermal cycling for 1 cycle of 96° C. for 15 sec to denature, then 15 cycles of 94° C. for 15 sec to denature and 65° C. for 60 sec to anneal and extend.

An equal volume of PCR buffer containing a high concentration of the two pairs of universal primers (25 pmol of each primer; Table 2) and 1.3 units of Amplitaq DNA polymerase Stoffel fragment was added to the Microamp reaction tube, and thermal cycling was performed for another 25 cycles with the annealing temperature lowered to 55° C. The upstream universal primers ALg1 and CLg3 were fluorescently labeled with 6-FAM and TET, respectively. All thermal cycling was achieved with a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems).

The primary PCR process was carried out under the following conditions:

5.9 μl	H_2O
5 μl	primer pairs 1–12 (2 pmol each primer)
2.5 μl	10X Stoffel fragment
4 μl	25 mM $MgCl_2$
5 μl	2 mM dNTP stock (each)
2.5 μl	genomic DNA (10 ng)
0.13 μl	Stoffel Frag. (1.3 units)
25 μl	Total

The primary PCR cycling conditions were as follows:

96° C. 15"

94° C. 15", 65° C. 1'×15

65° C. Hold

The secondary PCR process was carried out under the following conditions:

13.37 μl	H_2O
5 μl	zip primer pairs (25 pmol each primer)
2.5 μl	10X Stoffel fragment
4 μl	25 mM $MgCl_2$
0.13 μl	Stoffel Frag. (1.3 units)
25 μl	Total

The secondary PCR cycling conditions were as follows:

94° C. 15", 55° C. 1'×25

4° C. Hold

The PCR products were separated on an Applied Biosystems 373 DNA sequencer. A 3 ml aliquot of PCR sample was mixed with 3 ml of formamide containing fluorescently labeled Genescan-2500 [TAMRA] size standard (Applied Biosystems). The formamide/standard solution was prepared by adding 50 ml Genescan-2500 size standard [TAMRA] to 450 ml of formamide. The sample was heated at 95° C. for 2 min, quick cooled in ice, and electrophoresed through a denaturing 8% polyacrylamide gel in an Applied Biosystems 373 DNA sequencer running Genescan version 1.2 software. The sizes of the fluorescently labeled products were automatically computed by the Genescan analysis software using the local Southern method. The electropherograms clearly showed 12 distinct products (FIG. **28**). The uniform amount of each product was attributed to the similar size of each amplicon and the use of the primary primers with portions complementary to the secondary primers, which annealed with identical affinities to the 12 amplicons without the need to carefully adjust reaction conditions. The computed sizes of the products, which ranged from 135 to 175 bp, matched exactly to their actual sizes (see Table 12).

TABLE 12

List of PCR and LDR Products

Site	PCR Products		LDR Product			
Number	Label	Size (bp)	Label	Size (bp)	Variation	Allele
1	6-FAM	143	6-FAM	49	C	A
			TET		T	B
2	TET	145	6-FAM	35	C	A
			TET		T	B
3	6-FAM	147	6-FAM	43	A	A
			TET		C	B
4	TET	149	6-FAM	51	T	A
			TET		G	B
5	6-FAM	155	HEX	56	A	A
				58	G	B
6	TET	157	6-FAM	53	A	A
			TET		G	B
7	6-FAM	159	6-FAM	47	G	A
			TET		A	B
8	TET	161	6-FAM	45	G	A
			TET		A	B
9	TET	165	6-FAM	55	A	A
			TET		G	B
10	6-FAM	167	HEX	48	T	A
				50	G	B
11	TET	173	HEX	44	C	A
				46	T	B
12	6-FAM	175	HEX	40	G	A
				42	A	B

The dual labeling approach (Table 12) made it much easier to distinguish the products on the electropherograms (FIG. 28, compare panel A with panels B and C).

Example 10

Multiplex Ligase Detection Reaction

To avoid any possibility of labeled PCR product interfering with the detection of ligation product sequences, PCR product amplified using unlabeled PCR universal primers served as the target for the LDR. The polymerase in the PCR was inactivated by either freeze-thawing or adding EDTA/proteinase K to a final concentration of 5 mM and 100 mg/ml, respectively, and heating to 37° C. for 30 min and 95° C. for 10 min.

Proteinase K digestion was carried out under the following conditions:

20 μl	PCR product
2.5 μl	500 mg/ml proteinase K +
25 μl	Total

60° C. for 60 min.

95° C. for 10 min to heat kill proteinase K.

Four microliters of PCR product was diluted in 20 μl of LDR mix containing 50 mM Tris/HCl pH 8.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM NAD+, 10 mM DTT, LDR oligonucleotide sets 1–12 (200 fmol of each oligonucleotide), and 10 units of *Thermus aquaticus* DNA ligase (Barany, F. and Gelfand, D., "Cloning, Overexpression, and Nucleotide Sequence of a Thermostable DNA Ligase-Encoding Gene," *Gene,* 109:1–11 (1991), which is hereby incorporated by reference). Each LDR oligonucleotide probe set consisted of two allele-specific oligonucleotides and a common oligonucleotide. Each pair of discriminating allele-specific oligonucleotide probes in the LDR oligonucleotide probe sets 1, 2, 3, 4, 6, 7, 8, and 9 were the same size, with one oligonucleotide labeled with 6-FAM and the other labeled with TET. For LDR oligonucleotide probe sets 5, 10, 11, and 12, each pair of allele-specific oligonucleotides differed by 2 bases (the larger oligonucleotide had a 5' tail that was not complementary to the target sequence), and both oligonucleotides were labeled with HEX. Thermal cycling was performed for 1 cycle of 95° C. for 2 min to denature, then 20 cycles of 95° C. for 30 sec to denature, and 65° C. for 4 min to ligate.

The LDR process was carried out as follows:

4 μl	PCR product
2 μl	LDR oligo sets 1–12 (200 fmol each oligo)
2 μl	10X T. ligase buffer
2 μl	10 mM NAD+
1 μl	200 mM DTT
9 μl	H_2O
20 μl	Total +
1 μl	Taq Ligase (10 units)

Stock of Ligase: 0.5 μl of 625 u/ml+3.2 μl 10×buffer+27.5 μl H_2O

LDR cycling conditions were as follows:

94° C. 2'

94° C. 30", 65° C. 4'×20

4° C. Hold

A 3 μl aliquot of LDR sample was mixed with 3 μl of formamide containing fluorescently labeled Genescan-2500 [TAMRA] size standard (Applied Biosystems). The sample was heated at 95° C. for 2 min, quick cooled in ice, and electrophoresed through a denaturing 10% polyacrylamide gel in an Applied Biosystems 373 DNA sequencer running Genescan version 1.2 software. The sizes of the fluorescently labeled products were automatically computed by the Genescan analysis software using the local Southern method. LDR profiles of two individuals are shown in FIG. **29**. When each fluorescent dye was analyzed independently (FIG. **29**, panels B–D and F–H), it was very easy to determine the alleles present for each locus. A simple "A" or "B" code was assigned to each LDR product (Table 12) and used to score the genotypes. See Table 13 as follows:

TABLE 13

Genotypes Determined by PCR-LDR for 5 Individuals

	Individual Site Number											
	1	2	3	4	5	6	7	8	9	10	11	12
1	AB	AB	AB	AB	AB	AB	AB	AA	AA	AB	AB	AB
2	AA	BB	AB	BB	AB	AB	BB	AA	AB	AA	AA	AA
3	AB	AB	AB	BB	AA	BB	BB	AA	AA	AB	AB	AA
4	AA	AB	AA	AB	AB	AA	AB	AA	AB	BB	AB	AA
5	AA	AA	AA	AB	AB	AB	AB	AB	AA	AB	AB	BB

The first individual (FIG. **29**A–D; Table 13, individual 1) was heterozygous at polymorphic sites 1–7, and 10–12. Heterozygosity at sites 1–4 and 6–7 was indicated by the detection of both 6-FAM and TET labeled products (FIG. **29**, panels B and C) at the respective positions on the electropherograms. Heterozygosity at sites 5 and 11–12 was indicated by the presence of two HEX labeled products, differing in size by 2 bases, for each of these loci (FIG. **29**, panel D). In contrast, the one product detected at sites 8 and 9 (FIG. **29**, panels B and C) established that each of these loci was homozygous. The second individual (FIG. **29**E–F and G–H; Table 13, individual 2) was heterozygous only at sites 3, 5, 6, and 9 and homozygous at sites 1, 2, 4, 7, 8, 10–12. There was a total of 8 differences in the genotypes at these positions between the two persons. Three additional individuals were typed, and all 5 persons had distinct genotypes based on the 12 loci (Table 13).

Although the invention has been described in detail for the purpose of illustration, it is understood that such details are solely for that purpose. The variations can be made therein by those skilled in the art without departing from the spirit of the scope of the invention which is defined by the following claims.

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<220> FEATURE:
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Sequence

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<223> OTHER INFORMATION: Description of Artificial Sequence: Primer Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Primer Sequence

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<220> FEATURE:
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Primer Sequence

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<400> SEQUENCE: 76

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What is claimed:

1. A method for identifying one or more different target nucleotide sequences comprising:

providing a sample potentially containing one or more target nucleotide sequences comprising sequence differences;

providing one or more oligonucleotide probe sets, each set comprising (a) a first oligonucleotide probe comprising a target-specific portion and a 5' upstream primer-specific portion and (b) a second oligonucleotide probe comprising a target-specific portion and a 3' downstream primer-specific portion, wherein the first and second oligonucleotide probes in each particular set are suitable for ligation together when hybridized on a corresponding target nucleotide sequence, but have a mismatch which interferes with such ligation when first and second oligonucleotide probes are hybridized to any other nucleotide sequence present in the sample;

providing a ligase;

blending the sample, the one or more oligonucleotide probe sets, and the ligase to form a ligase detection reaction mixture;

subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles to form a ligation product sequence comprising (a) the 5' upstream primer specific portion, (b) the target-specific portions, and (c) the 3' downstream primer-specific portion, when the respective target nucleotide sequence of the corresponding oligonucleotide probe set is present in the sample;

providing one or a plurality of oligonucleotide primer sets, each set comprising (a) an upstream primer containing the same sequence as the 5' upstream primer-specific portion of the ligation product sequence and (b) a downstream primer complementary to the 3' downstream primer-specific portion of the ligation product sequence;

providing a polymerase;

blending the ligase detection reaction mixture with the one or a plurality of oligonucleotide primer sets, and the polymerase to form a polymerase chain reaction mixture;

subjecting the polymerase chain reaction mixture to one or more polymerase chain reaction cycles to form extension products comprising the ligation product sequence and/or complements thereof; and

detecting the extension products to identify one or more target nucleotide sequences in the sample.

2. A method according to claim **1**, wherein one of the oligonucleotide probes in each set comprises a restriction site, said method further comprising:

restriction digesting each extension product at the restriction site to produce extension product fragments, wherein the restriction site is positioned in each of the oligonucleotide probe sets to produce an extension product fragment with a unique length so that it can be distinguished from other nucleicacids in the polymerase chain reaction mixture after said restriction digesting; and

separating the extension product fragments by size or electrophoretic mobility, wherein said detecting differentiates the extension product fragments which differ in size.

3. A method according to claim **1**, wherein the ligation product sequence of the oligonucleotide probes in each particular set produces an extension product of unique length, said method further comprising:

separating the extension products by size or electrophoretic mobility, wherein said detecting differentiates the extension products which differ in size.

4. A method according to claim **1**, wherein the oligonucleotide probes in each set are configured so that the sequence of the ligation product sequence from each oligonucleotide probe set is unique and can be distinguished from other nucleic acids in the polymerase chain reaction mixture, said method further comprising:

providing a solid support with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the unique nucleotide sequences of given probe sets;

contacting the polymerase chain reaction mixture, after said subjecting it to one or more polymerase chain reaction cycles, with the solid support under conditions effective to hybridize the extension products to the capture oligonucleotides in a base-specific manner, wherein said detecting indicates the presence of extension products captured using the unique nucleotide sequence portions to identify one or more target nucleotide sequences in the sample.

5. A method according to claim **1**, wherein, in each primer set, one primer comprises a detectable reporter label and the other primer comprises an addressable array-specific portion which is linked to the 5' end of that primer and remains single stranded after said subjecting the polymerase chain reaction mixture to one or more polymerase chain reaction cycles, said method further comprising:

providing a solid support with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the addressable array-specific portions;

contacting the polymerase chain reaction mixture, after said subjecting it to one or more polymerase chain reaction cycles, with the solid support under conditions effective to hybridize the extension products to the capture oligonucleotides in a base-specific manner, wherein said detecting indicates the presence of extension products captured at particular sites to identify one or more target nucleotide sequences in the sample.

6. A method according to claim **1**, wherein the relative amounts of two or more differing sequences are present in a sample in unknown amounts with a plurality of target nucleotide sequences being quantified and a set of oligonucleotide primers being useful in amplifying all the ligation product sequences formed by the oligonucleotide probe sets in each particular probe group, the oligonucleotide probe sets forming a plurality of oligonucleotide probe groups, each group comprised of two or more of the oligonucleotide probe sets, wherein oligonucleotide probe sets in the same group comprise the same 5' upstream primer-specific portion and the same 3' downstream primer-specific portion, said method further comprising:

quantifying the relative amount of the extension products, after said detecting and

comparing relative amounts of the extension products generated to provide a quantitative measure of the relative level of the two or more target nucleotide sequences in the sample.

7. A method according to claim **6**, wherein one of the oligonucleotide probes in each set comprises a restriction site, said method further comprising:

restriction digesting the extension products at the restriction site to produce extension product fragments, wherein the restriction site is positioned in each of the oligonucleotide probe sets to produce an extension product fragment with a unique length so that it can be distinguished from other nucleic acids in the polymerase chain reaction mixture after said restriction digesting; and

separating the extension product fragments by size or electrophoretic mobility, wherein said detecting is carried out by size differences in the extension product fragments.

8. A method according to claim **6**, wherein oligonucleotide probe sets in the same group comprise the same 5' upstream primer-specific portion and the same 3' downstream primer-specific portion, and the ligation product sequences of oligonucleotide probes in each particular set have a unique length product so that they can be distinguished from other nucleic acids in the polymerase chain reaction mixture, said method further comprising:

separating the extension products by size or electrophoretic mobility, wherein said detecting is carried out by size differences in the extension products.

9. A method according to claim **6**, wherein the ligation product sequences of oligonucleotide probes in each particular set comprise unique sequences so that they can be distinguished from other nucleic acids in the polymerase chain reaction mixture, said method further comprising:

providing a solid support with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the unique nucleotide sequences of given probe sets;

contacting the polymerase chain reaction mixture, after said subjecting it to one or more polymerase chain reaction cycles, with the solid support under conditions effective to hybridize the extension products to the capture oligonucleotides in a base-specific manner; and

detecting the presence of extension products captured at particular sites.

10. A method according to claim **1**, wherein one or both oligonucleotide probes in each particular set comprise blocking groups at their non-ligating ends with blocking group rendering the ligation product sequence of the oligonucleotide probes in a particular set substantially resistant to exonuclease digestion, said method further comprising:

subjecting the ligase detection reaction mixture to exonuclease digestion after said subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles and

inactivating the exonuclease.

11. A method according to claim **10**, wherein the ligation product sequence of the oligonucleotide probes in each particular set generates an extension product of unique length, said method further comprising:

separating the extension products by size or electrophoretic mobility, wherein said detecting differentiates the extension products which differ in size.

12. A method according to claim **11**, wherein the one or more target nucleotide sequences are present in the sample

in unknown amounts with a plurality of target nucleotide sequences being quantified, said method further comprising:

providing a known amount of one or more marker target nucleotide sequences;

providing one or a plurality of oligonucleotide probe groups, each group comprised of two or more of the oligonucleotide probe sets, comprising probe sets specifically designed for the marker target nucleotide sequences, wherein one or both oligonucleotide probes in each particular set are blocked at their non-ligating ends, wherein oligonucleotide probe sets in the same group comprise either (a) the same 5' upstream primer-specific portion or (b) the same 3' downstream primer-specific portion, or (c) both the same 5' upstream primer-specific portion and the same 3' downstream primer-specific portion, said ligase detection reaction mixture further comprising the marker target nucleotide sequences and the probe sets including probe sets specifically designed for the marker target nucleotide sequences, said method further comprising:

providing one or a plurality of oligonucleotide primer groups, each group comprised of two or more oligonucleotide primer sets, wherein the oligonucleotide primer sets in each group comprise either the same 5' upstream primer or the same 3' downstream primer or both the same 5' upstream primer and the same 3' downstream primer, a group of oligonucleotide primers being useful to amplify all ligation product sequences in a given group;

quantifying the amount of extension products after said detecting; and

comparing the amounts of extension products generated from the unknown sample with the amount of extension products generated from known amounts of marker target nucleotide sequences to provide a quantitative measure of the level of one or more target nucleotide sequences in the sample.

13. A method according to claim **10**, wherein one primer comprises a detectable reporter label and the other primer comprises an addressable array-specific portion which is linked to the 5' end of that primer and remains single stranded after said subjecting the polymerase chain reaction mixture to one or more polymerase chain reaction cycles, said method further comprising:

providing a solid support with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the addressable array-specific portions and

contacting the polymerase chain reaction mixture, after said subjecting it to one or more polymerase chain reaction cycles, with the solid support under conditions effective to hybridize the extension products to the capture oligonucleotides in a base-specific manner, wherein said detecting indicates the presence of extension products captured at particular sites to identify one or more target nucleotide sequences in the sample.

14. A method according to claim **13**, wherein the one or more different target sequences are present in the sample in unknown amounts with a plurality of target nucleotide sequences being quantified, one primer comprising a detectable reporter label and the other primer comprising an addressable array-specific portion which is linked to the 5' end of that primer and remains single stranded after said subjecting the polymerase chain reaction mixture to one or more polymerase chain reaction cycles, and the oligonucleotide primer sets in the same group comprise either the same 5' upstream primer or the same 3' downstream primer, wherein a group of oligonucleotide primers may be used to amplify all ligation product sequences in a given group, said method further comprising:

providing a known amount of one or more marker target nucleotide sequences;

providing one or a plurality of oligonucleotide probe groups, each group comprised of two or more of the oligonucleotide probe sets, including probe sets specifically designed for the marker target nucleotide sequences, wherein one or both oligonucleotide probe sets in the same group comprise either the same 5' upstream primer-specific portion or the same 3' downstream primer-specific portion;

providing one or a plurality of oligonucleotide primer groups, each group comprised of two or more oligonucleotide primer sets, wherein the oligonucleotide primer sets in the same group comprise either the same 5' upstream primer or the same 3' downstream primer and a group of oligonucleotide primers are used to amplify all ligation product sequences in a group;

blending the marker target nucleotide sequences and probe sets specifically designed for the marker target nucleotide sequences with the ligase detection reaction mixture;

quantifying the amount of extension products; and

comparing the amounts of extension products generated from the unknown sample with the amount of extension products generated from known amounts of marker target nucleotide sequences to provide a quantitative measure of the level of one or more target nucleotide sequences in the sample.

15. A method according to claim **1**, wherein one or both oligonucleotide probes in each particular set comprise deoxy-uracil in place of deoxy-thymidine with the deoxy-uracil rendering the oligonucleotide probes and their ligation product sequences substantially sensitive to uracil N-glycosylase, said method further comprising:

blending the ligase detection reaction mixture, after said subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles and before said subjecting the polymerase chain reaction mixture to one or more polymerase chain reaction cycles, with one or a plurality of the downstream primers complementary to the 3' downstream primer-specific portion of the ligation product sequences, and the polymerase to form an extension mixture;

subjecting the extension mixture to a hybridization treatment, wherein the downstream primer hybridizes to the 3' downstream primer-specific portion of the ligation product sequence and extends to form an extension product complementary to the ligation product sequence;

inactivating the polymerase;

blending the extension mixture, after said inactivating, with uracil N-glycosylase to form a uracil N-glycosylase digestion mixture;

subjecting the extension mixture to uracil-N-glycosylase digestion substantially to destroy oligonucleotide probes, ligation product sequences, and extension products generated from original target which use the 5' upstream primer as primers, without destroying the 3' downstream primer extension product generated from the ligation product sequences;

inactivating the uracil N-glycosylase;

blending, after said inactivating the uracil N-glycosylase, a polymerase with the uracil N-glycosylase digestion mixture to form the polymerase chain reaction mixture;

subjecting the polymerase chain reaction mixture to the one or more polymerase chain reaction cycles to form an extension product in the first cycle which is substantially the same as the ligation product sequence except comprising deoxy-thymidine in place of deoxy-uracil, and, in subsequent cycles, the 5' upstream primer hybridizes to the 5' upstream primer-specific portion of the extension product complementary to the ligation product sequence and the 3' downstream primer hybridizes to the 3' downstream portion of the extension product sequence which is substantially the same as the ligation product sequence, an extension treatment, whereby said subjecting the extension mixture to uracil N-glycosylase digestion substantially reduces the quantity of the ligation product sequences, either one or both oligonucleotide probes, and, thus, ligation independent extension products from said subjecting the polymerase chain reaction mixture to one or more polymerase chain reaction cycles.

16. A method according to claim **15**, wherein the ligation product sequences of oligonucleotide probes in a particular set generates a unique length product which is distinguishable from either probes or other ligation product sequences, said method further comprising:

separating the extension products by size or electrophoretic mobility, wherein said detecting differentiates the extension products which differ in size.

17. A method according to claim **16**, wherein the one or more target nucleotide sequences are present in the sample in unknown amounts with a plurality of target nucleotide sequences being quantified, said method further comprising:

providing a known amount of one or more marker target nucleotide sequences;

providing one or more marker-specific oligonucleotide probe sets, each set comprising (a) a first oligonucleotide probe comprising a target-specific portion and a 5' upstream primer-specific portion and (b) a second oligonucleotide probe comprising a target-specific portion and a 3' downstream primer-specific portion, wherein one or both oligonucleotide probes in each particular set comprise deoxy-uracil in place of deoxy-thymidine, wherein the oligonucleotide probes in each particular set are suitable for ligation together when hybridized to a corresponding marker target nucleotide sequence, but, when hybridized to any other nucleotide sequence present in the sample or added marker sequences, have a mismatch which interferes with such ligation, said oligonucleotide probe sets and said marker-specific oligonucleotide sets forming a plurality of oligonucleotide probe groups, wherein the ligation product sequence of oligonucleotide probes in each particular set generates a unique length product, and thus may be distinguished from either probes or other ligation product sequences in the same group or other groups;

blending the marker target nucleotide sequences and the probe sets specifically designed for the marker target nucleotide sequences with the ligase detection mixture;

providing one or a plurality of oligonucleotide primer groups, each group comprised of two or more of the oligonucleotide primer sets, wherein oligonucleotide primer sets in the same group comprise either the same 5' upstream primer or the same 3' downstream primer, or both the same 5' upstream primer and the same 3' downstream primer, wherein a group of oligonucleotide primers may be used to amplify all the ligation product sequences in a given group;

separating the extension products by size or electrophoretic mobility;

quantifying the amount of extension products after said detecting; and

comparing the amounts of extension products generated from the unknown sample with the amount of extension products generated from known amounts of marker target nucleotide sequences to prove a quantitative measure of the level of one or more target nucleotide sequences in the sample.

18. A method according to claim **15**, wherein, in each primer set, one primer comprises a detectable reporter label and the other primer comprises an addressable array-specific portion which is linked to the 5' end of that primer and remains single stranded after said subjecting the polymerase chain reaction mixture of one or more polymerase chain reaction cycles, said method further comprising:

providing a solid support with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the array-specific portions and

contacting the polymerase chain reaction mixture, after said subjecting it to one or more polymerase chain reaction cycles, with the solid support under conditions effective to hybridize the extension products to the capture oligonucleotides in a base-specific manner, wherein said detecting indicates the presence of extension products.

19. A method according to claim **18**, wherein the one or more different target nucleotide sequences are present in the sample in unknown amounts with a plurality of target nucleotide sequences being quantified, said method further comprising:

providing a known amount of one or more marker target nucleotide sequences;

providing one or a plurality of oligonucleotide probe groups, each group comprised of two or more of the oligonucleotide probe sets, including probe sets specifically designed for the marker target nucleotide sequences, wherein the ligation product sequences of oligonucleotide probes in each particular set may be distinguished from either probes or other ligation product sequences in the same group or other groups;

blending the marker target nucleotide sequences and the probe sets specifically designed for the marker target nucleotide sequences with the ligase detection mixture;

providing one or a plurality of oligonucleotide primer groups, each group comprised of two or more of the oligonucleotide primer sets, wherein oligonucleotide primer sets in the same group comprise either the same 5' upstream primer or the same 3' downstream primer, wherein a group of oligonucleotide primers may be used to amplify all ligation product sequences in a given group;

quantifying the amount of extension products; and

comparing the amounts of extension products generated from the unknown sample with the amount of extension products generated from known amounts of marker target nucleotide sequences to provide a quantitative

measure of the level of one or more target nucleotide sequences in the sample.

20. A method according to claim **1**, wherein each cycle of the ligase detection reaction is from about 30 seconds to about five minutes long.

21. A method according to claim **1**, wherein said subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles is repeated for 2 to 50 cycles.

22. A method according to claim **1**, wherein total time for said subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles is 1 to 250 minutes.

23. A method according to claim **1**, wherein the ligase is selected from the group consisting of *Thermus aquaticus* ligase, *Thermus thermophilus* ligase, *E. coli* ligase, T4 ligase, and Pyrococcus ligase.

24. A method according to claim **1**, wherein the target-specific portions of the oligonucleotide probes each have a hybridization temperature of 50–50° C.

25. A method according to claim **1**, wherein the target-specific portions of the oligonucleotide probes are 20 to 28 nucleotides long.

26. A method according to claim **1**, wherein the oligonucleotide probe sets are selected from the group consisting of ribonucleotides, deoxyribonucleotides, modified ribonucleotides, modified deoxyribonucleotides, modified phosphate-sugar backbone oligonucleotides, nucleotide analogues, and mixtures thereof.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

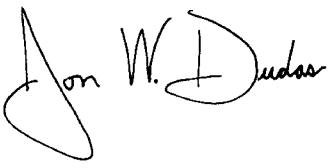
PATENT NO. : 6,797,470 B2
APPLICATION NO. : 09/918156
DATED : September 28, 2004
INVENTOR(S) : Barany et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 1 at lines 13-15, delete "This invention was developed with government funding under National lnstitutes of Health Grant No. GM41337-06. The U.S. Government may retain certain rights." and insert --This invention was made with government support under grant GM41337-06 awarded by the National lnstitutes of Health. The government has certain rights in the invention-- in its place.

Signed and Sealed this

Seventh Day of October, 2008



JON W. DUDAS
Director of the United States Patent and Trademark Office

Exhibit B

US007083917B2

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

(10) **Patent No.: US 7,083,917 B2**
(45) **Date of Patent: *Aug. 1, 2006**

(54) **DETECTION OF NUCLEIC ACID SEQUENCE DIFFERENCES USING THE LIGASE DETECTION REACTION WITH ADDRESSABLE ARRAYS**

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

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/963,920**

(22) Filed: **Sep. 26, 2001**

(65) **Prior Publication Data**

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(60) Provisional application No. 60/011,359, filed on Feb. 9, 1996.

(51) **Int. Cl.**

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C07H 21/02	(2006.01)
G01N 33/543	(2006.01)

(52) **U.S. Cl.** **435/6**; 435/91.1; 435/91.52; 435/DIG. 1; 435/DIG. 14; 435/DIG. 17; 435/DIG. 22; 435/DIG. 37; 436/501; 436/518; 536/23.1; 536/24.3

(58) **Field of Classification Search** ..... 435/6, 435/91.1, 91.52, DIG. 2, DIG. 14, DIG. 17, 435/DIG. 22, DIG. 37, 285.1; 436/501, 436/518; 536/23.1, 24.3

See application file for complete search history.

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Assistant Examiner—Amber D. Steele
(74) *Attorney, Agent, or Firm*—Nixon Peabody LLP

(57) **ABSTRACT**

The present invention describes a method for identifying one or more of a plurality of sequences differing by one or more single base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. The method includes a ligation phase, a capture phase, and a detection phase. The ligation phase utilizes a ligation detection reaction between one oligonucleotide probe, which has a target sequence-specific portion and an addressable array-specific portion, and a second oligonucleotide probe, having a target sequence-specific portion and a detectable label. After the ligation phase, the capture phase is carried out by hybridizing the ligated oligonucleotide probes to a solid support with an array of immobilized capture oligonucleotides at least some of which are complementary to the addressable array-specific portion. Following completion of the capture phase, a detection phase is carried out to detect the labels of ligated oligonucleotide probes hybridized to the solid support. The ligation phase can be preceded by an amplification process. The present invention also relates to a kit for practicing this method, a method of forming arrays on solid supports, and the supports themselves.

19 Claims, 34 Drawing Sheets

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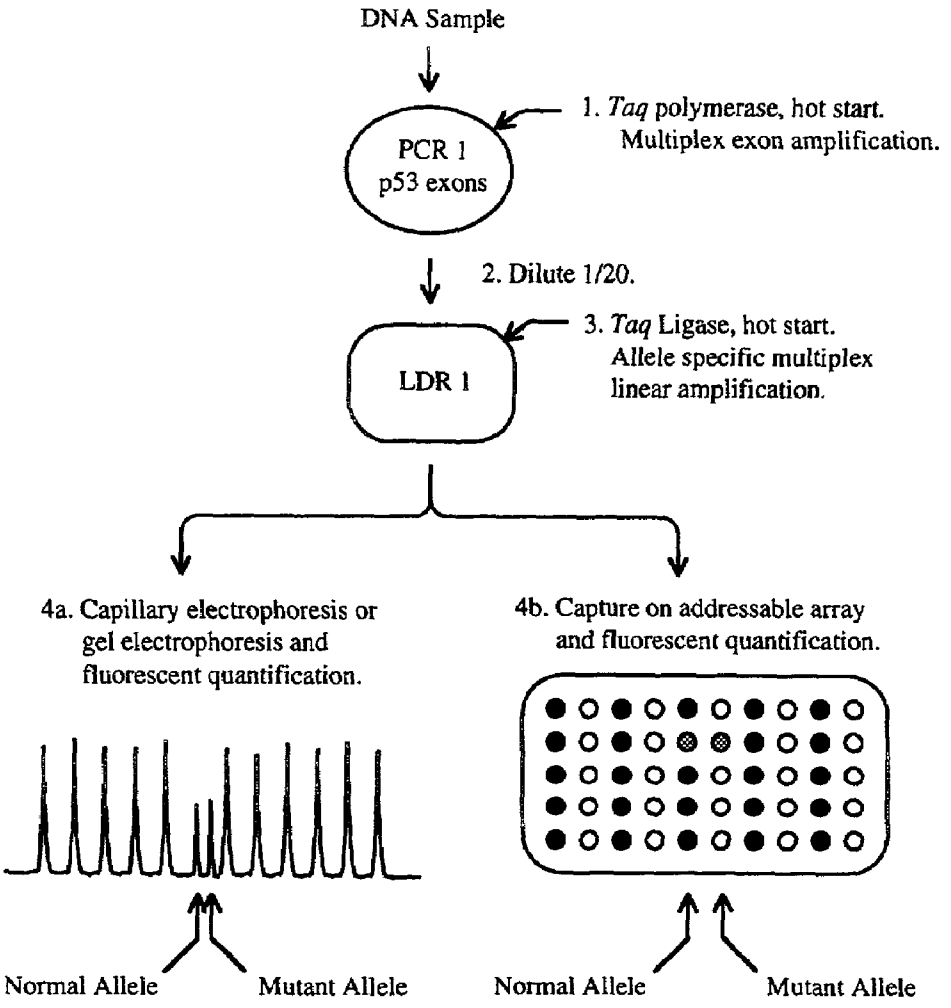

DNA Sample
1. *Taq* polymerase, hot start. Multiplex exon amplification.
PCR 1 p53 exons
2. Dilute 1/20.
3. *Taq* Ligase, hot start. Allele specific multiplex linear amplification.
LDR 1
4a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
4b. Capture on addressable array and fluorescent quantification.
Normal Allele
Mutant Allele
Normal Allele
Mutant Allele

FIG. 1

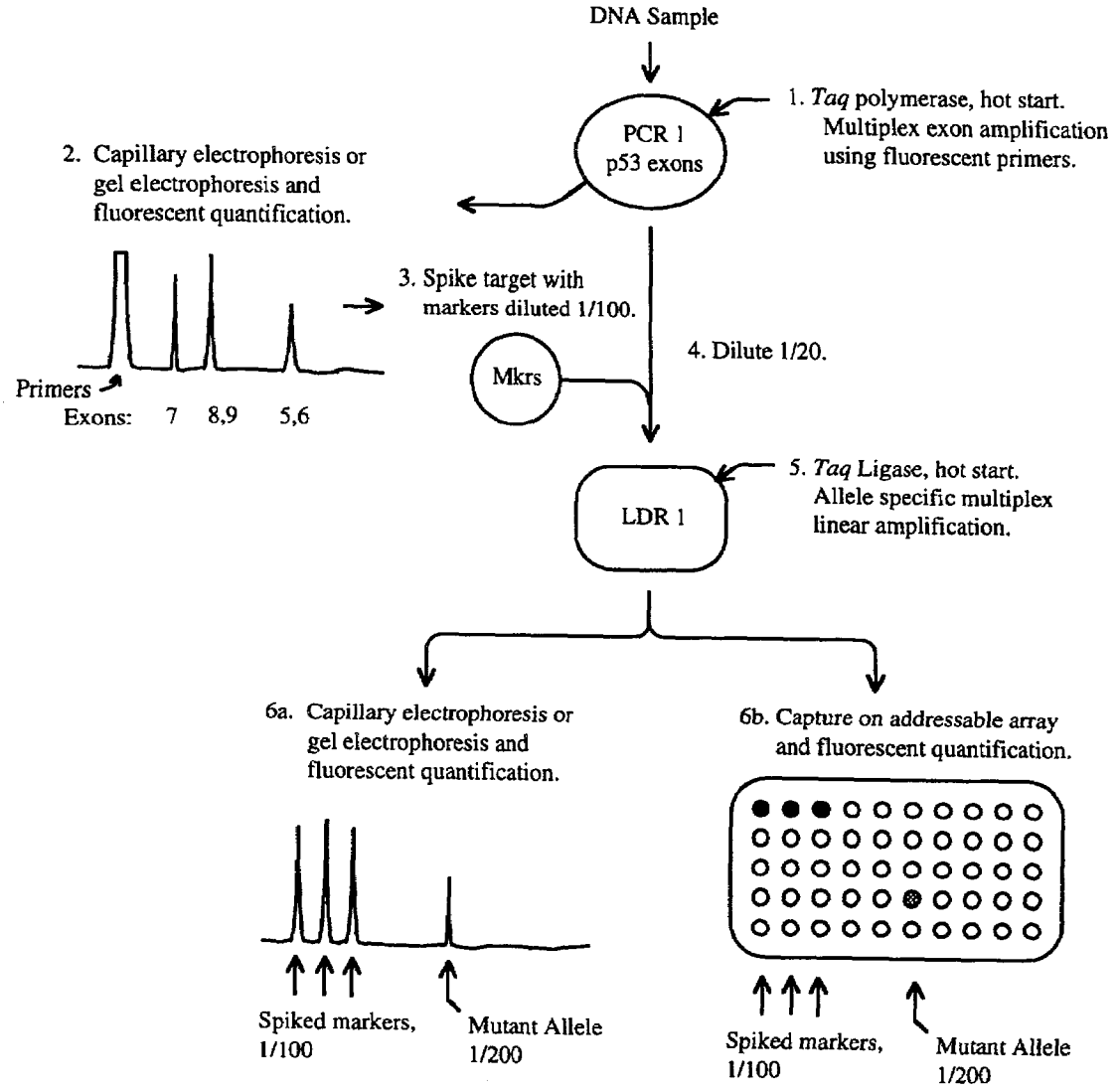

DNA Sample
1. *Taq* polymerase, hot start. Multiplex exon amplification using fluorescent primers.
PCR 1 p53 exons
2. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
3. Spike target with markers diluted 1/100.
Mkrs
4. Dilute 1/20.
Primers
Exons: 7 8,9 5,6
5. *Taq* Ligase, hot start. Allele specific multiplex linear amplification.
LDR 1
6a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
6b. Capture on addressable array and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200
Spiked markers, 1/100
Mutant Allele 1/200

FIG. 2

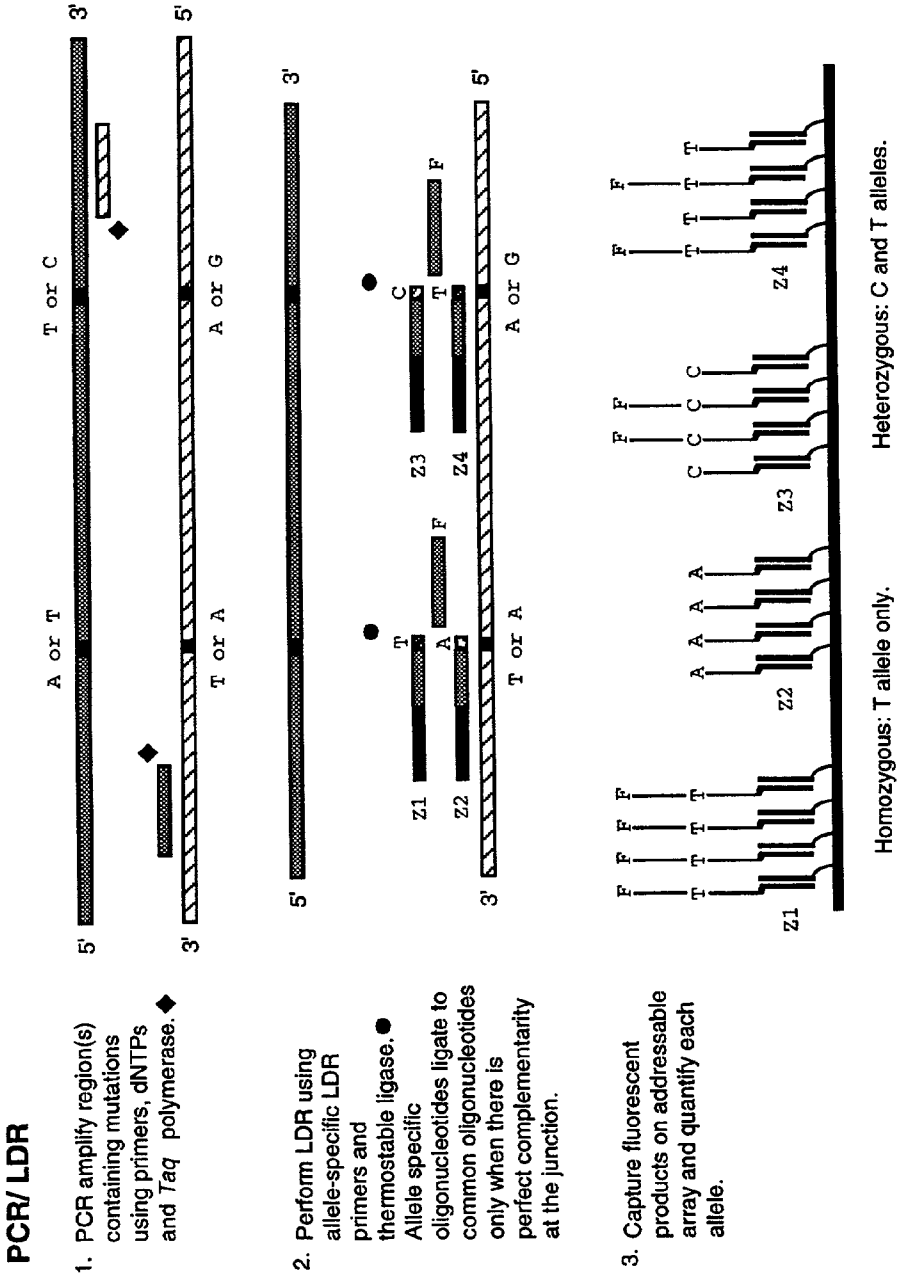

PCR/ LDR
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
3. Capture fluorescent products on addressable array and quantify each allele.
A or T
T or C
T or A
A or G
Z1
Z2
Z3
Z4
F
Homozygous: T allele only.
Heterozygous: C and T alleles.

FIG. 3

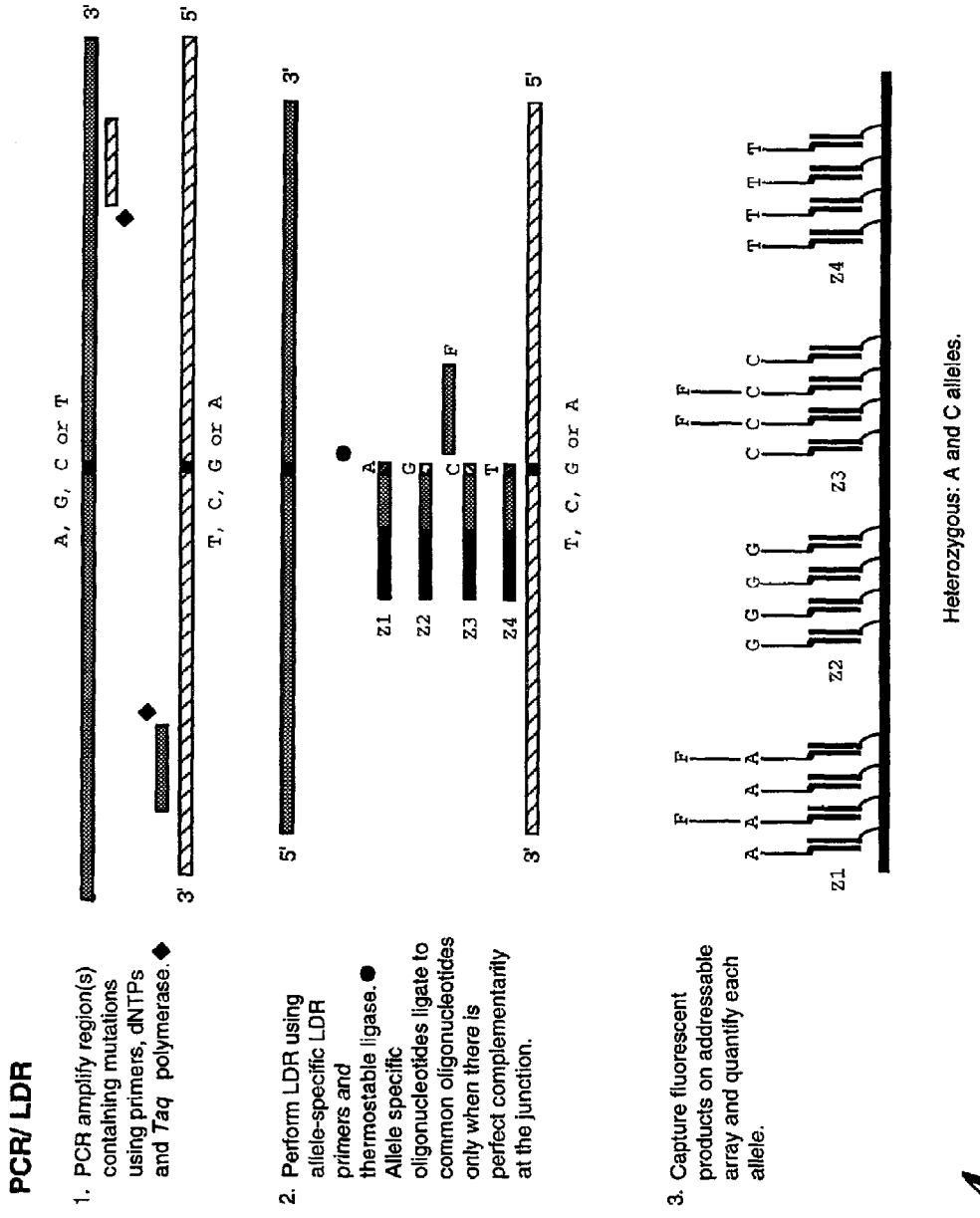

PCR/ LDR
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
A, G, C or T
T, C, G or A
3'
5'
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
Z1
Z2
Z3
Z4
A
G
C
T
F
3. Capture fluorescent products on addressable array and quantify each allele.
Heterozygous: A and C alleles.

FIG. 4

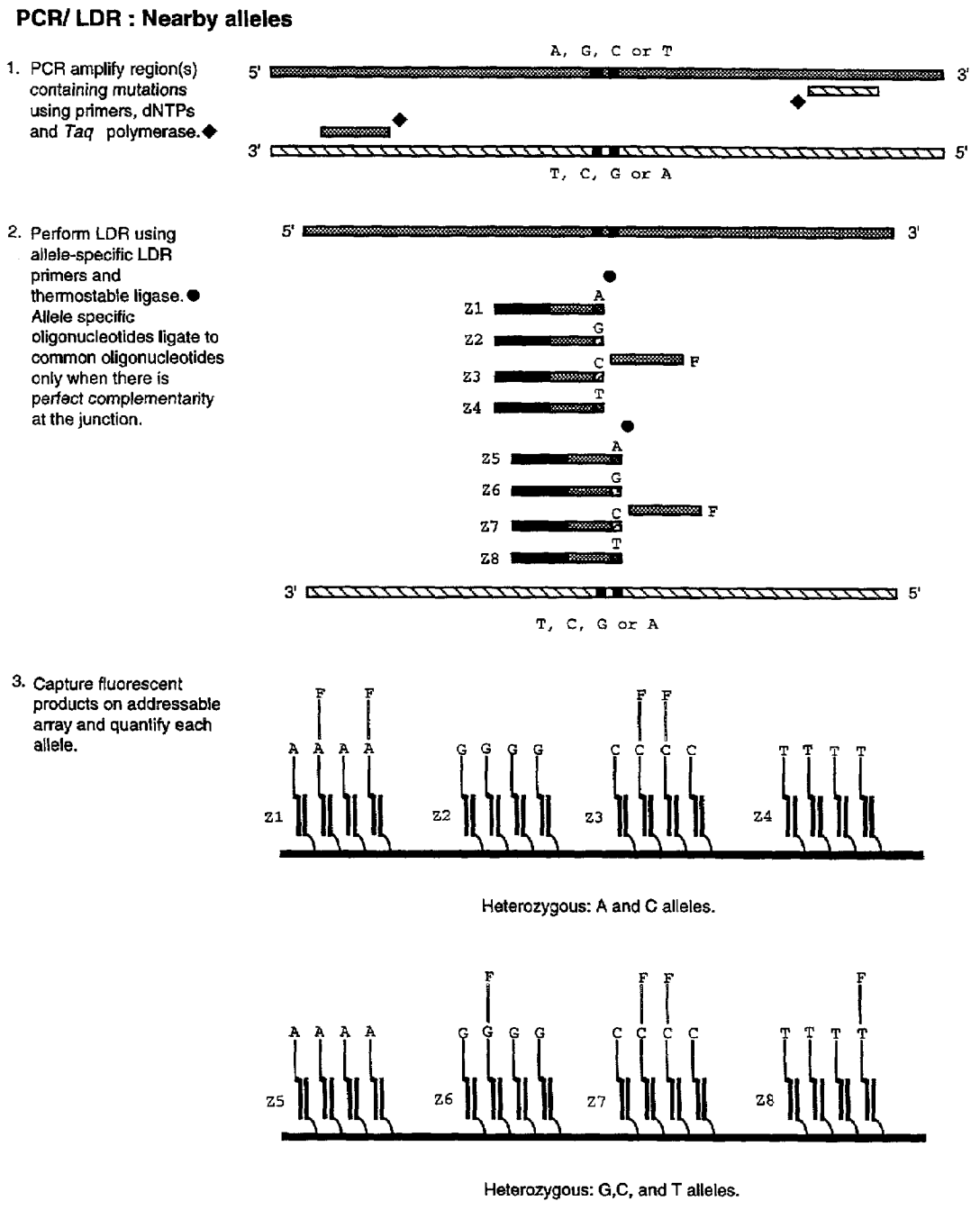

PCR/ LDR : Nearby alleles
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
A, G, C or T
5'
3'
3'
5'
T, C, G or A
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
5'
3'
Z1
Z2
Z3
Z4
Z5
Z6
Z7
Z8
A
G
C
T
F
3'
5'
T, C, G or A
3. Capture fluorescent products on addressable array and quantify each allele.
Z1
Z2
Z3
Z4
Heterozygous: A and C alleles.
Z5
Z6
Z7
Z8
Heterozygous: G,C, and T alleles.

FIG. 5

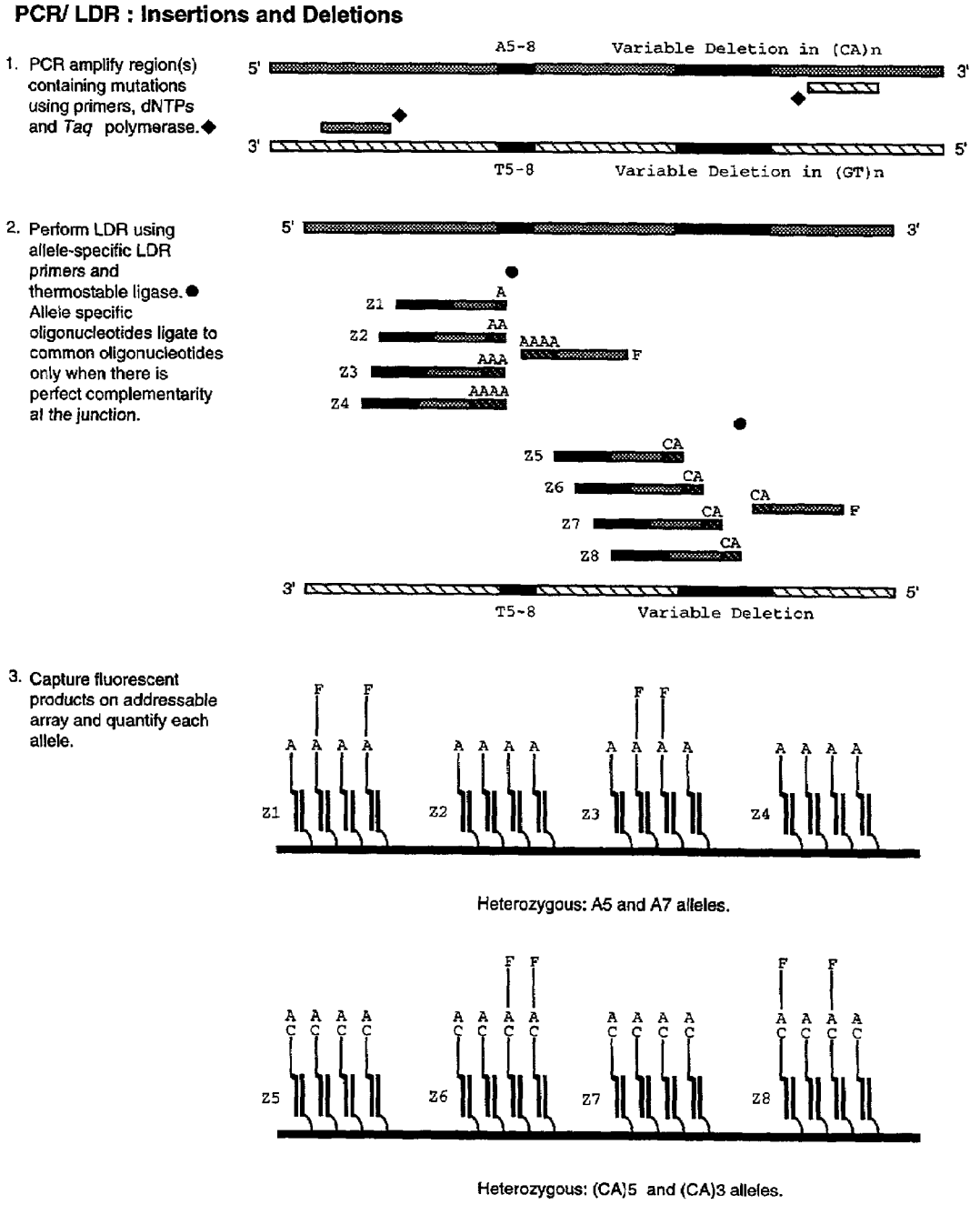

PCR/ LDR : Insertions and Deletions
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
A5-8
Variable Deletion in (CA)n
5'
3'
3'
5'
T5-8
Variable Deletion in (GT)n
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
Z1
Z2
Z3
Z4
Z5
Z6
Z7
Z8
F
T5-8
Variable Deletion
3. Capture fluorescent products on addressable array and quantify each allele.
Heterozygous: A5 and A7 alleles.
Heterozygous: (CA)5 and (CA)3 alleles.

FIG. 6

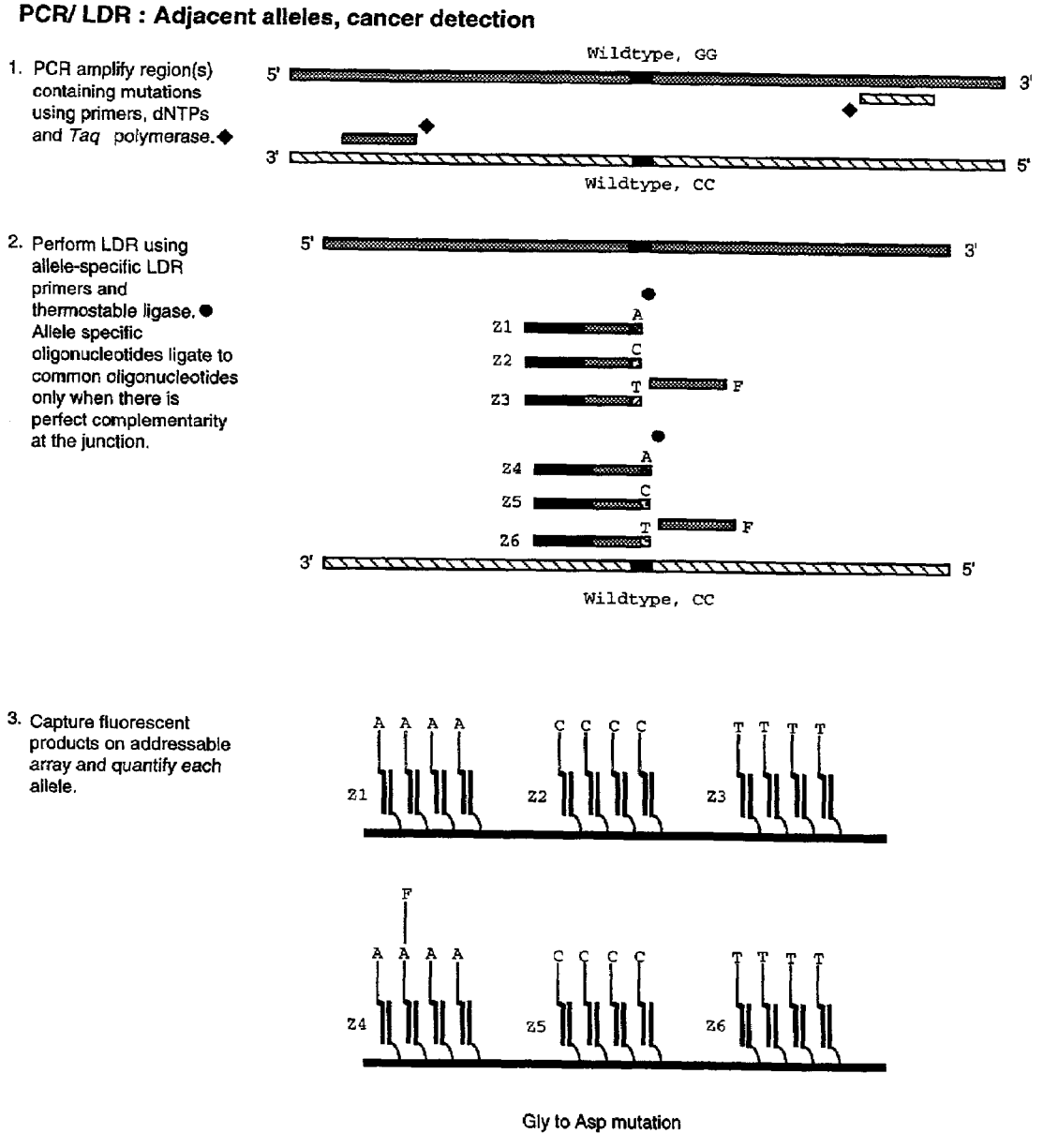

PCR/ LDR : Adjacent alleles, cancer detection
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
Wildtype, GG
5'
3'
3'
5'
Wildtype, CC
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
5'
3'
Z1
A
Z2
C
Z3
T
F
Z4
A
Z5
C
Z6
T
F
3'
5'
Wildtype, CC
3. Capture fluorescent products on addressable array and quantify each allele.
Z1
A A A A
Z2
C C C C
Z3
T T T T
F
Z4
A A A A
Z5
C C C C
Z6
T T T T
Gly to Asp mutation

FIG. 7

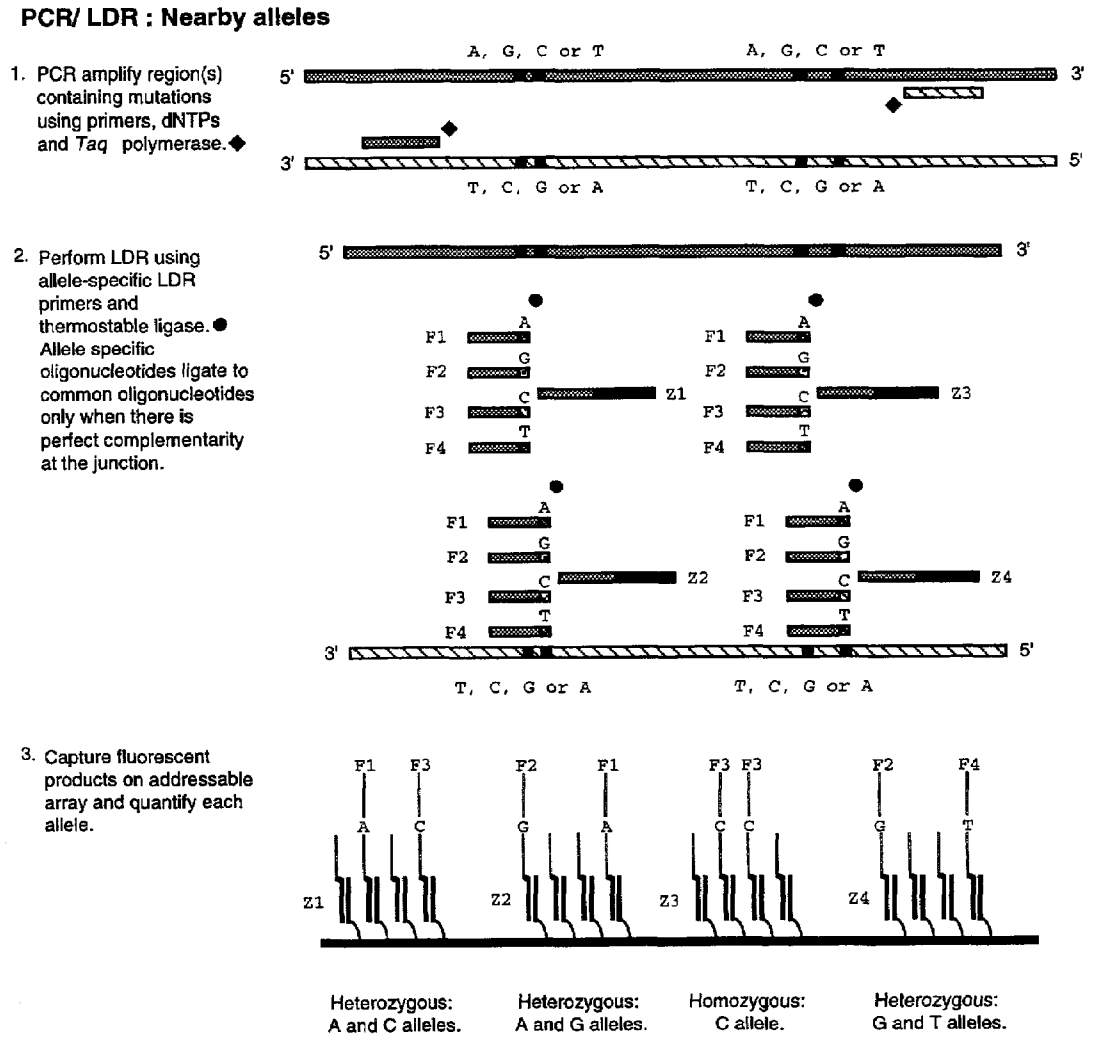

PCR/ LDR : Nearby alleles
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
3. Capture fluorescent products on addressable array and quantify each allele.
A, G, C or T
T, C, G or A
5'
3'
F1
F2
F3
F4
Z1
Z2
Z3
Z4
Heterozygous: A and C alleles.
Heterozygous: A and G alleles.
Homozygous: C allele.
Heterozygous: G and T alleles.

FIG. 8

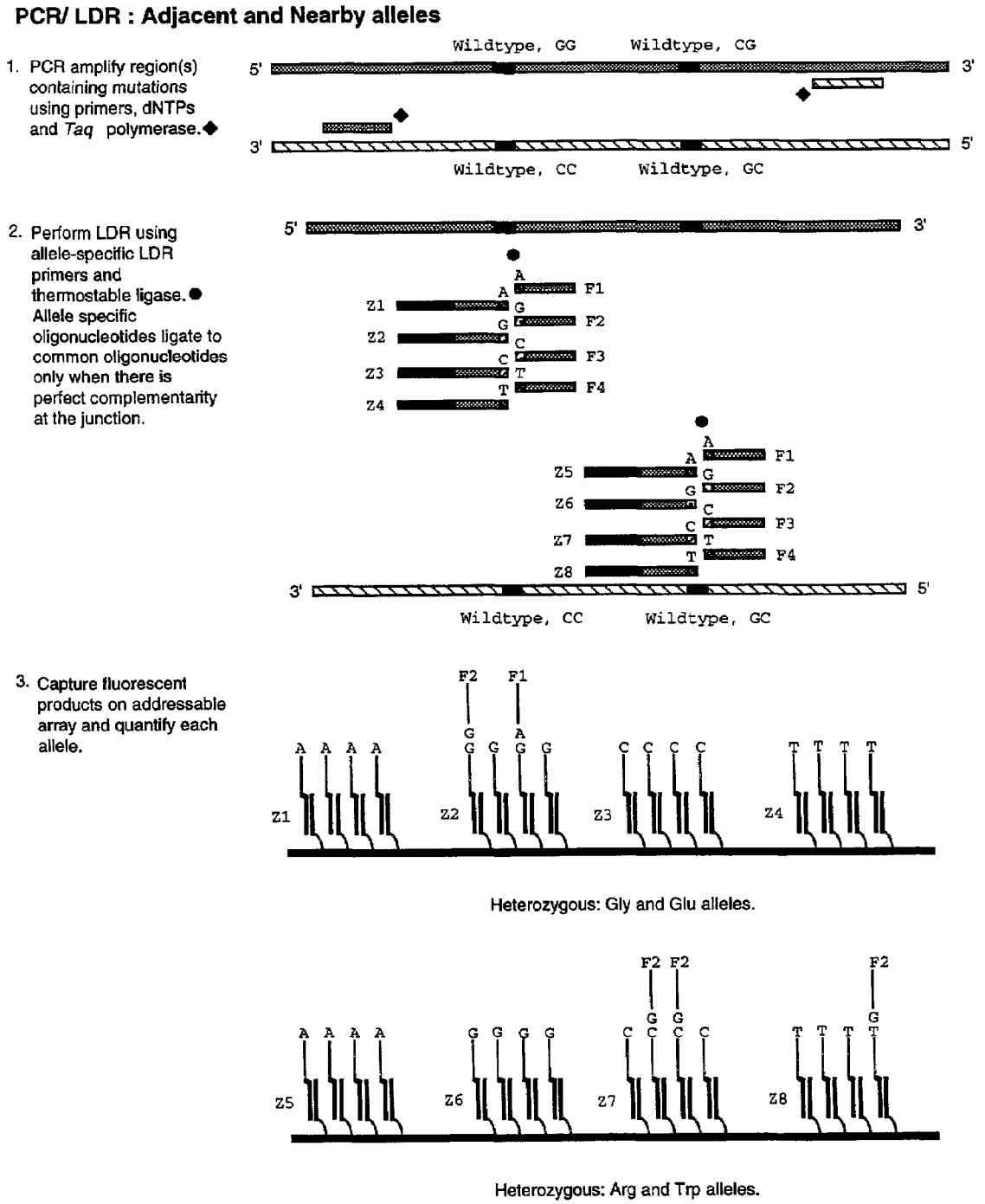

PCR/ LDR : Adjacent and Nearby alleles
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
Wildtype, GG
Wildtype, CG
Wildtype, CC
Wildtype, GC
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
Z1
Z2
Z3
Z4
Z5
Z6
Z7
Z8
F1
F2
F3
F4
3. Capture fluorescent products on addressable array and quantify each allele.
Heterozygous: Gly and Glu alleles.
Heterozygous: Arg and Trp alleles.

FIG. 9

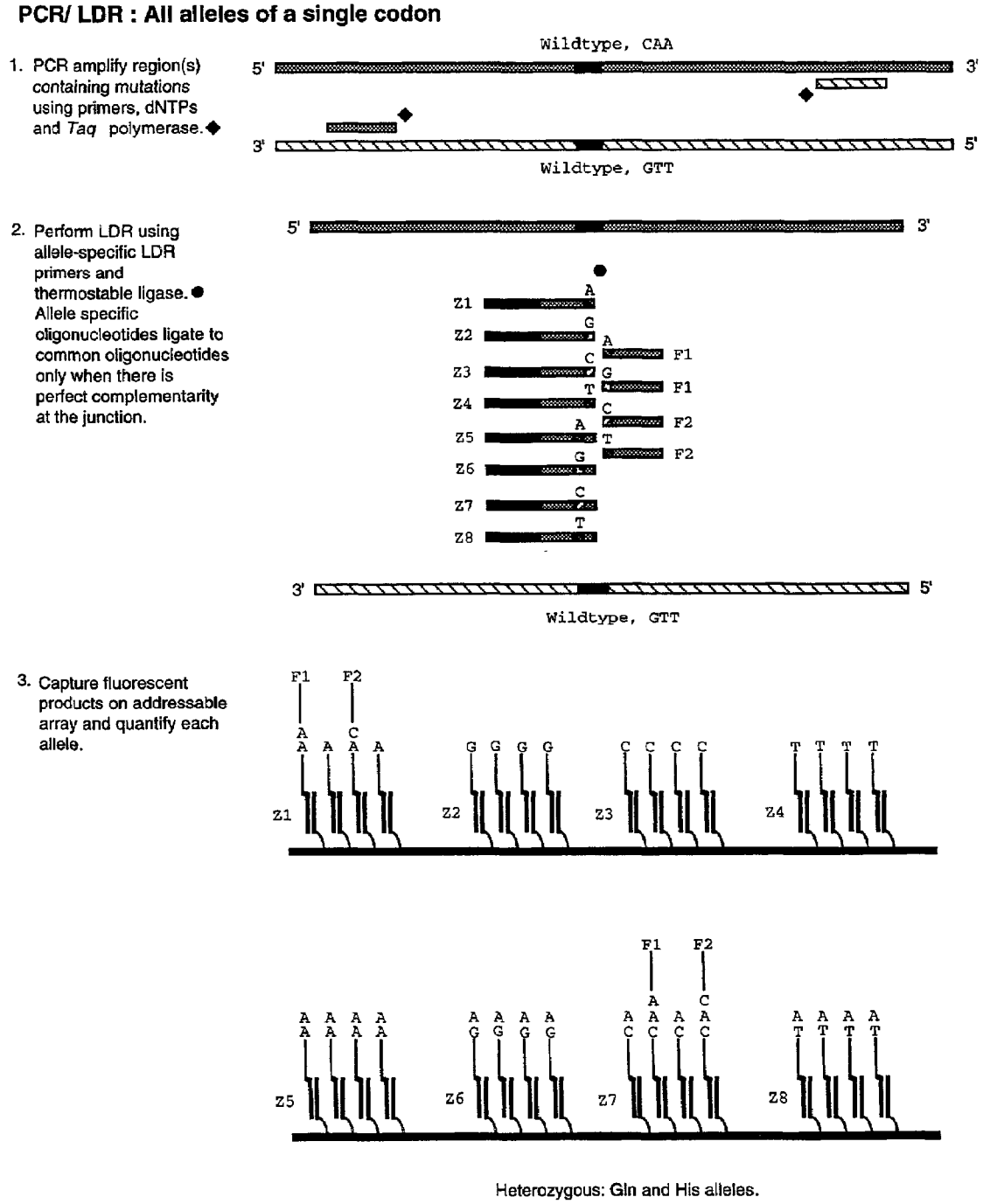
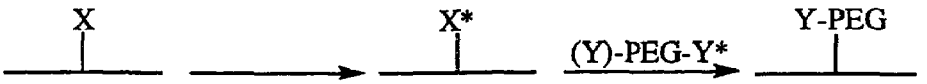

PCR/ LDR : All alleles of a single codon
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
Wildtype, CAA
5'
3'
3'
5'
Wildtype, GTT
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
5'
3'
Z1
Z2
Z3
Z4
Z5
Z6
Z7
Z8
A
G
A
C
G
T
C
A
T
G
C
T
F1
F1
F2
F2
3'
5'
Wildtype, GTT
3. Capture fluorescent products on addressable array and quantify each allele.
F1
F2
Z1
Z2
Z3
Z4
Z5
Z6
Z7
Z8
Heterozygous: Gln and His alleles.

FIG. 10



X,Y = –OH, –CO_2H, –NH_2
(Y) = W–NH–

X*, Y* = –O(C=O)Z, –O(C=S)Z, –CO_2H, –(C=O)Z, –NH_2, –N=C=O

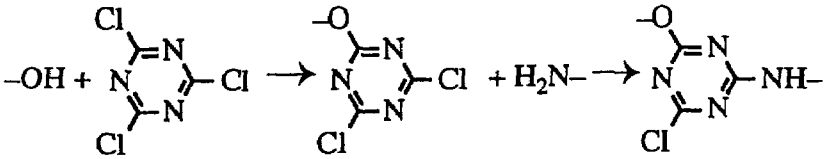
W = protecting group, e.g. Boc, Fmoc
Z = activating group, e.g. imidazole (Im), *p*-nitrophenol (OPnp), hydroxysuccinimide (OSu), pentafluorophenol (OPfp)
PEG = oligo or poly(ethylene glycol), backbone $(CH_2CH_2O)_n$ n = 6 to 200 (can also be grown by anionic polymerization with )
WSC = water soluble carbodiimide

Functional group transformations/activation (as needed), X → X*, Y → Y*

–OH ⟶ $–O(CH_2)_nCO_2H$ n = 1, 2
–OH ⟶ $–O(C=O)NHCH_2CO_2H$
–OH ⟶ $–O(C=O)CH_2NH_2$
–OH ⟶ –O(C=O)Im
–OH ⟶ $–O(C=S)SCH_2(C=O)NH_2$
$–CO_2H$ ⟶ $–(C=O)NH(CH_2)_nNH_2$ n = 2,6
$–CO_2H$ ⟶ –(C=O)Z
$–NH_2$ ⟶ $–NH(C=O)(CH_2)_nCO_2H$ n = 2, 3

Covalent linkage, X* + Y*

$–CO_2H + H_2N– + WSC + HOSu \longrightarrow –(C=O)NH–$
$–OH + Im(C=O)Im + H_2N– \longrightarrow –O(C=O)NH–$



$–OH + O=C=N– \longrightarrow –O(C=O)NH–$
$–O(C=S)SCH_2(C=O)NH_2 + H_2N– \longrightarrow –O(C=S)NH–$
–OH +  + HO– ⟶ $–OCH_2CH(OH)CH_2O–$ (+ $H_2NH–$) (NH–)
$–OH \longrightarrow –OCH_2(C=O)H + H_2N– + NaCNBH_3 \longrightarrow –OCH_2CH_2NH–$

FIG. 11

FIG. 12A

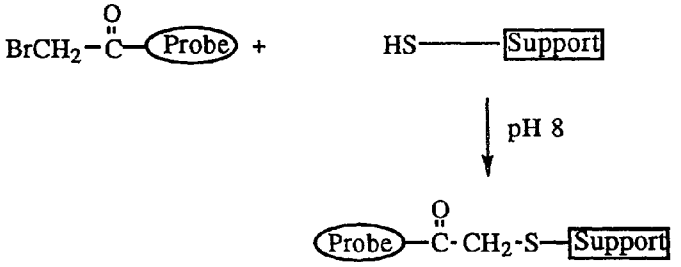


FIG. 12B

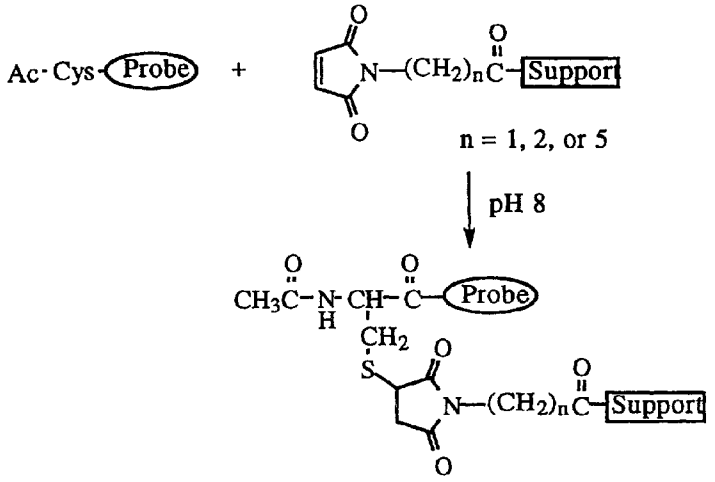
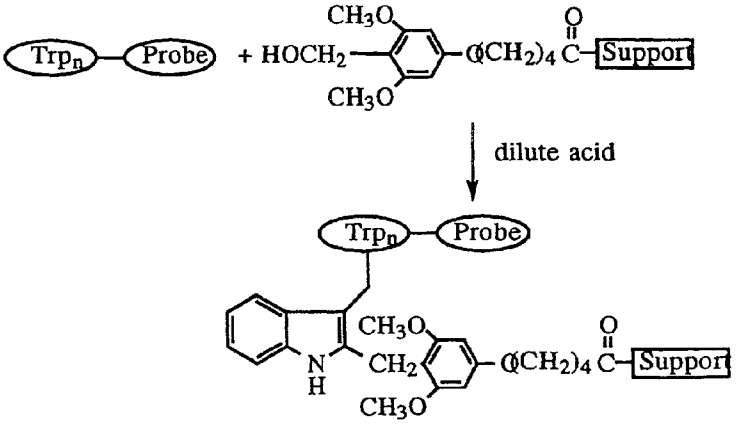


FIG. 12C



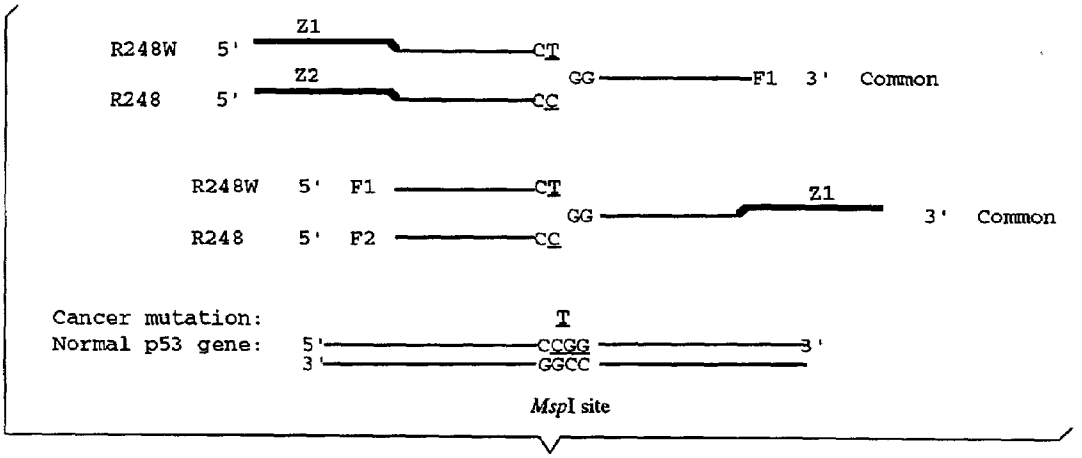


FIG. 13A

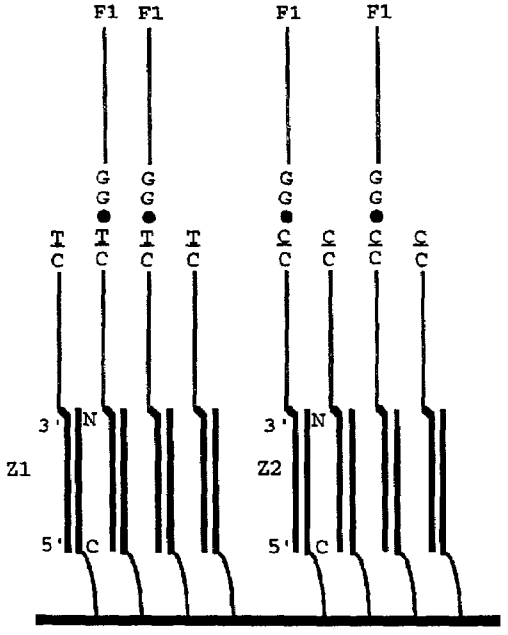


FIG. 13B

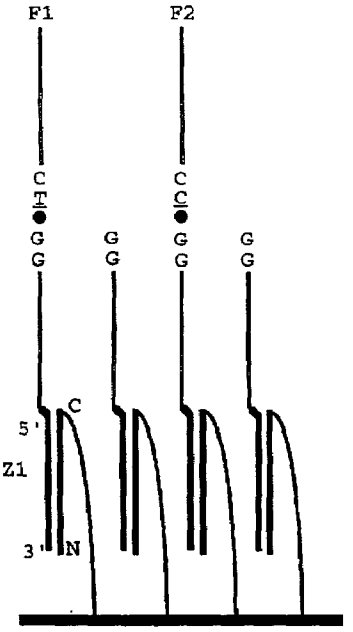


FIG. 13C

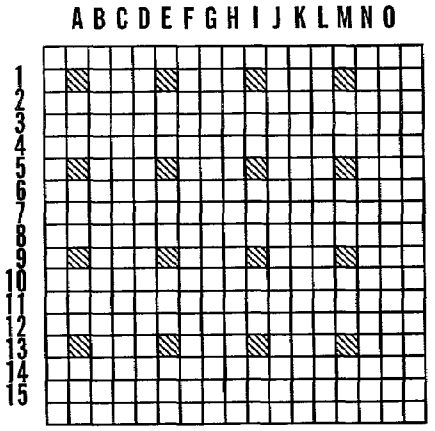


FIG. 14A

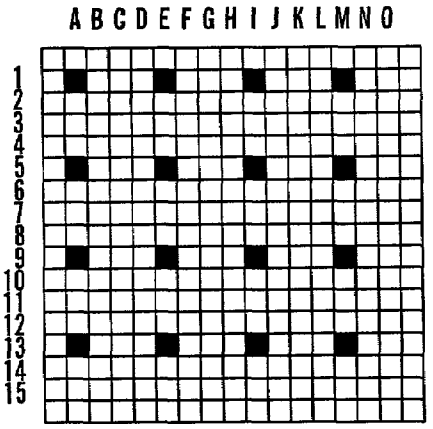


FIG. 14B

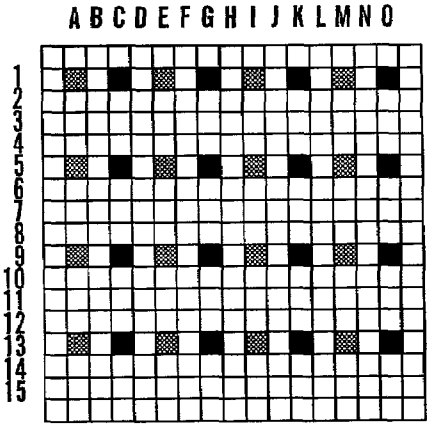


FIG. 14C

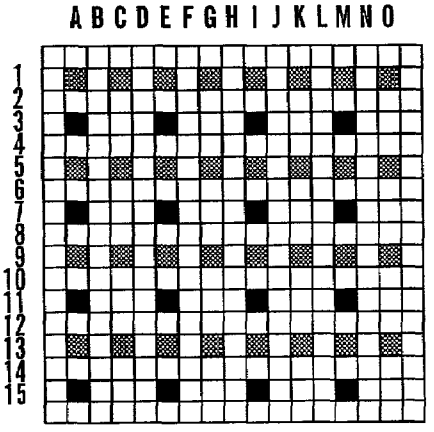


FIG. 14D

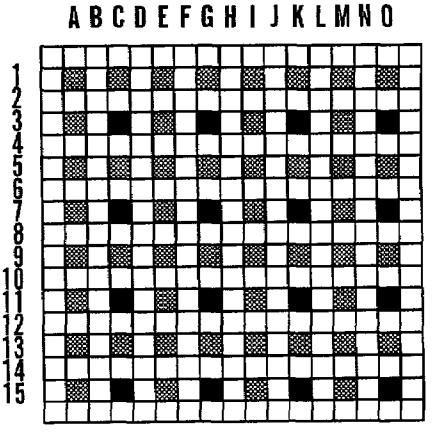


FIG. 14E

FIG. 15A

1st addition of unique 24mers.

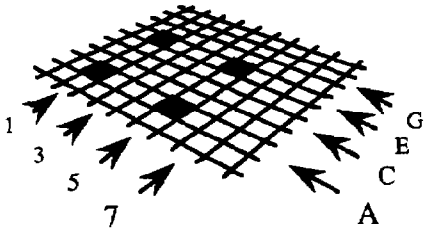


FIG. 15B

2nd addition of unique 24mers.

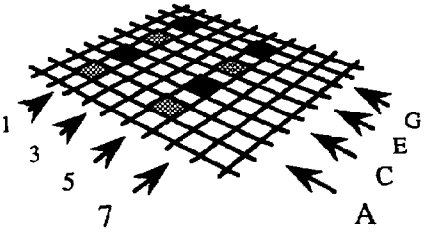


FIG. 15C

3rd addition of unique 24mers.

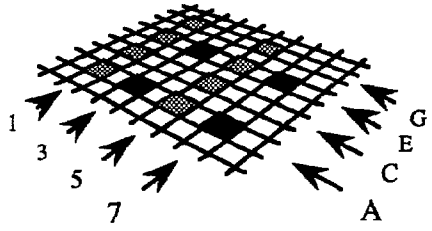
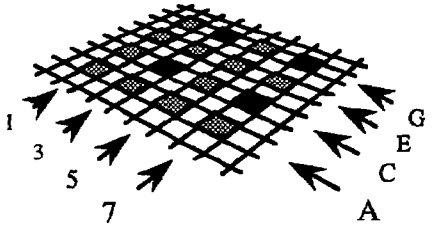


FIG. 15D

4th addition of unique 24mers.



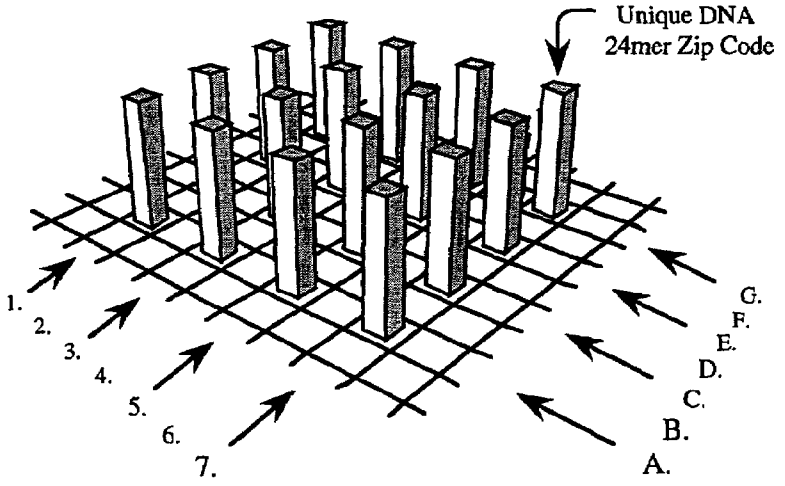


FIG. 15E

FIG. 16A

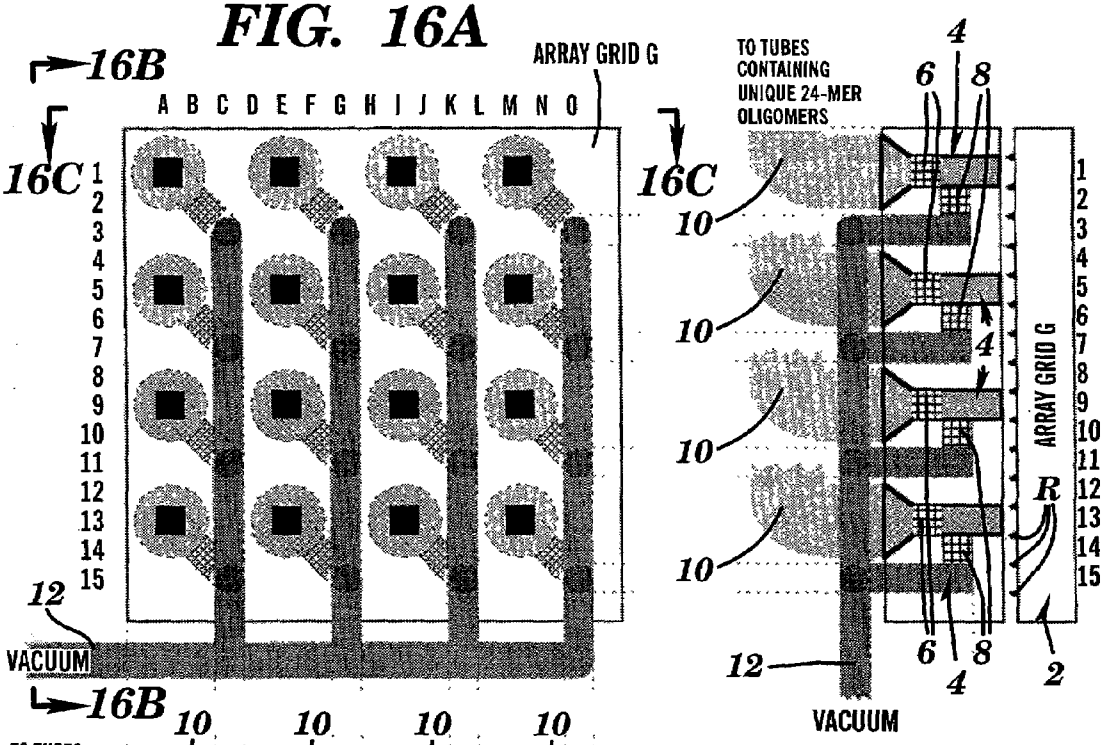


FIG. 16B

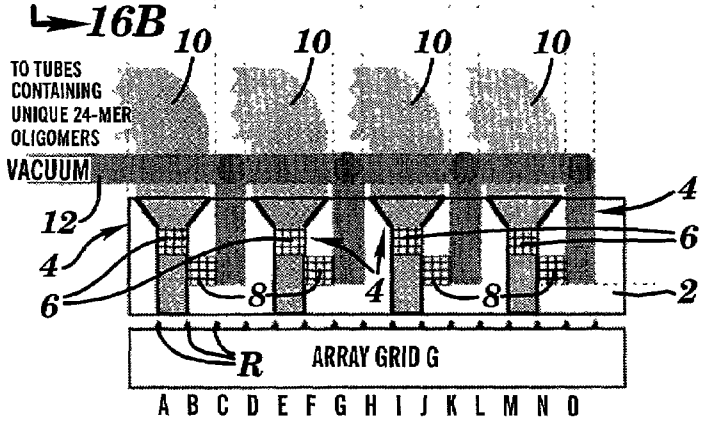


FIG. 16C

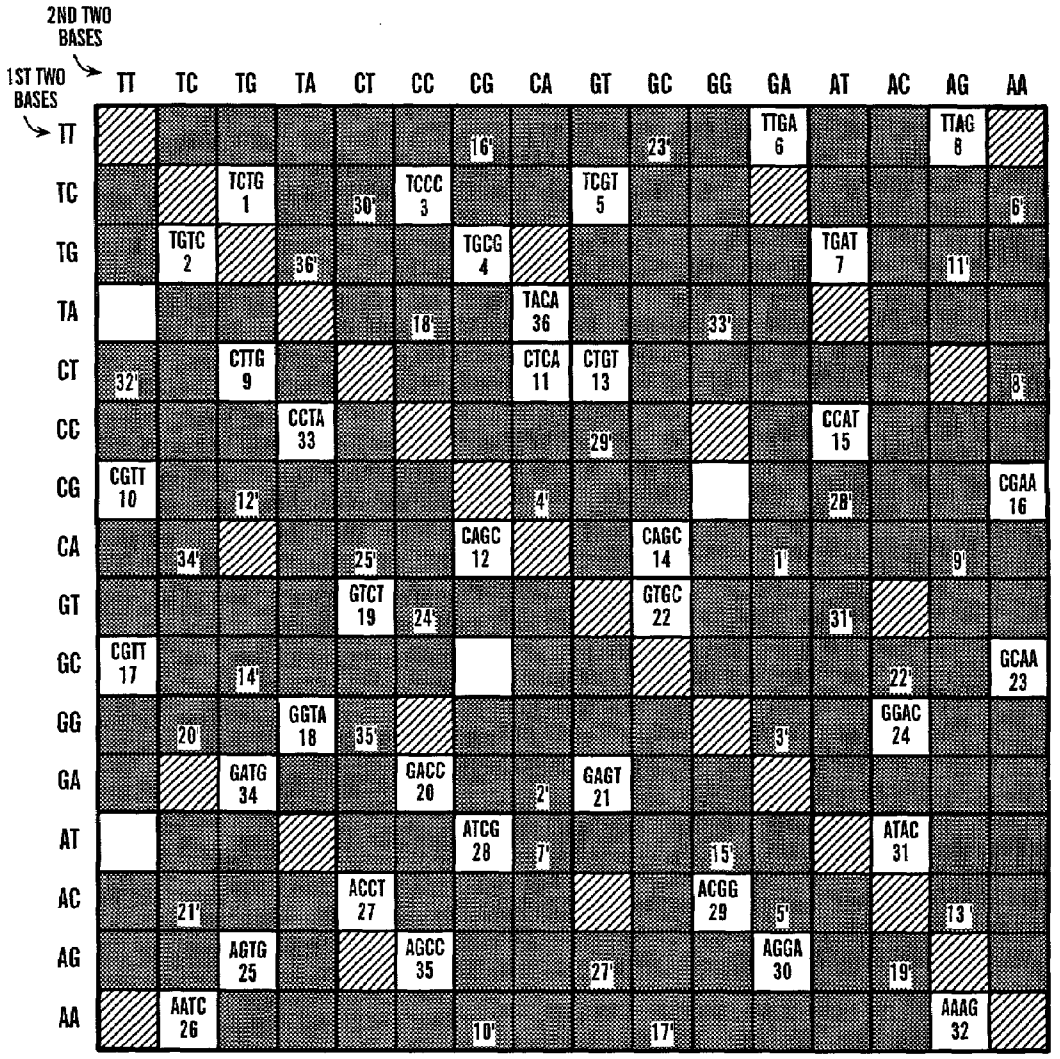

2ND TWO BASES
1ST TWO BASES
TT TC TG TA CT CC CG CA GT GC GG GA AT AC AG AA
TT
TC
TG
TA
CT
CC
CG
CA
GT
GC
GG
GA
AT
AC
AG
AA
TTGA 6
TTAG 8
TCTG 1
TCCC 3
TCGT 5
TGTC 2
TGCG 4
TGAT 7
TACA 36
CTTG 9
CTCA 11
CTGT 13
CCTA 33
CCAT 15
CGTT 10
CGAA 16
CAGC 12
CAGC 14
GTCT 19
GTGC 22
CGTT 17
GCAA 23
GGTA 18
GGAC 24
GATG 34
GACC 20
GAGT 21
ATCG 28
ATAC 31
ACCT 27
ACGG 29
AGTG 25
AGCC 35
AGGA 30
AATC 26
AAAG 32
16
23
30
36
18
33
32
8
29
12
4
34
25
1
9
24
31
14
22
20
35
3
2
7
15
21
13
27
19
10
17

FIG. 17

1st Tetramer addition (columns)

1	2	3	4	5
1	2	3	4	5
1	2	3	4	5
1	2	3	4	5
1	2	3	4	5

FIG. 18A

2nd Tetramer addition (rows)

6	6	6	6	6
5	5	5	5	5
4	4	4	4	4
3	3	3	3	3
2	2	2	2	2

FIG. 18B

3rd Tetramer addition (columns)

3	4	5	6	1
3	4	5	6	1
3	4	5	6	1
3	4	5	6	1
3	4	5	6	1

FIG. 18C

4th Tetramer addition (rows)

2	2	2	2	2
1	1	1	1	1
6	6	6	6	6
5	5	5	5	5
4	4	4	4	4

FIG. 18D

5th Tetramer addition (columns)

6	1	2	3	4
6	1	2	3	4
6	1	2	3	4
6	1	2	3	4
6	1	2	3	4

FIG. 18E

6th Tetramer addition (rows)

3	3	3	3	3
2	2	2	2	2
1	1	1	1	1
6	6	6	6	6
5	5	5	5	5

FIG. 18F

Addressable array with full length PNA 24mers

1-6-3-2-6-3	2-6-4-2-1-3	3-6-5-2-2-3	4-6-6-2-3-3	5-6-1-2-4-3
1-5-3-1-6-2	2-5-4-1-1-2	3-5-5-1-2-2	4-5-6-1-3-2	5-5-1-1-4-2
1-4-3-6-6-1	2-4-4-6-1-1	3-4-5-6-2-1	4-4-6-6-3-1	5-4-1-6-4-1
1-3-3-5-6-6	2-3-4-5-1-6	3-3-5-5-2-6	4-3-6-5-3-6	5-3-1-5-4-6
1-2-3-4-6-5	2-2-4-4-1-5	3-2-5-4-2-5	4-2-6-4-3-5	5-2-1-4-4-5

FIG. 18G

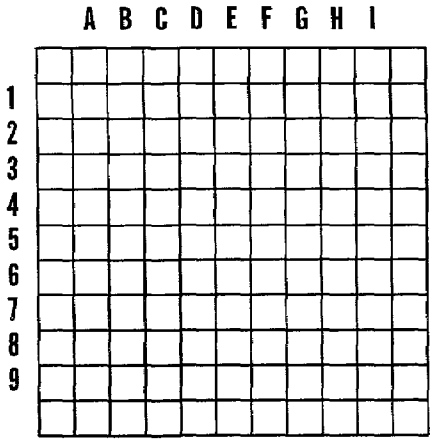


FIG. 19A

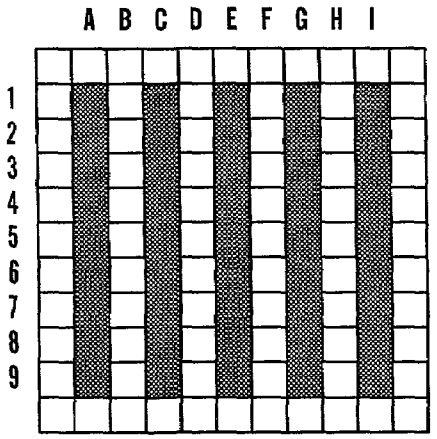


FIG. 19B

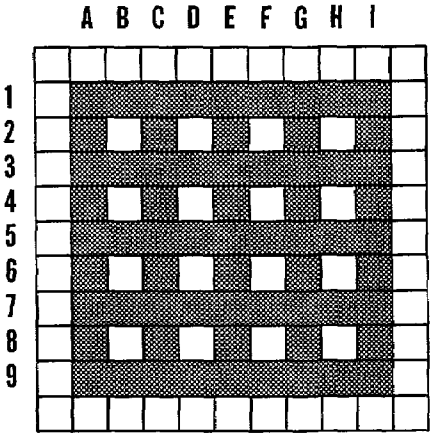


FIG. 19C

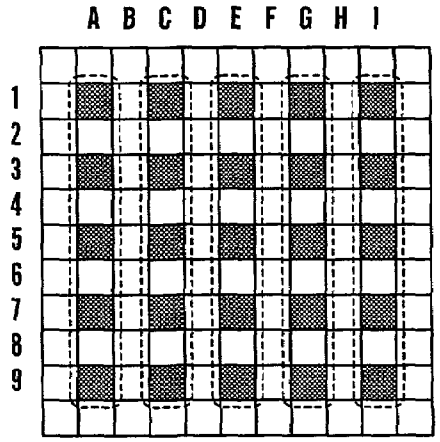


FIG. 19D

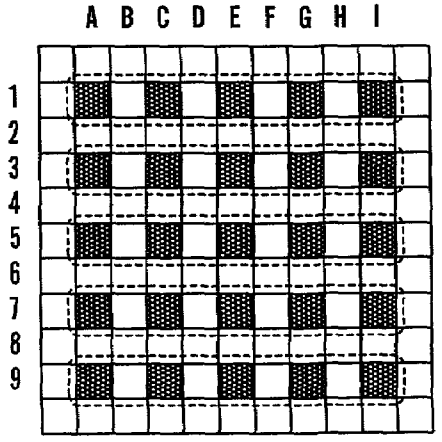


FIG. 19E

FIG. 20A

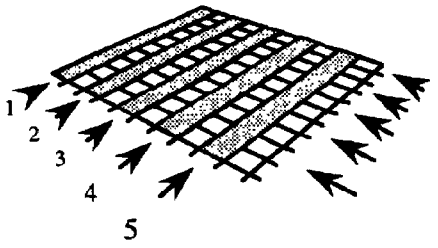
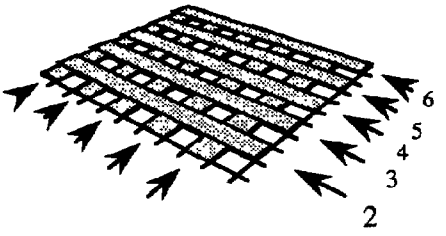


FIG. 20B



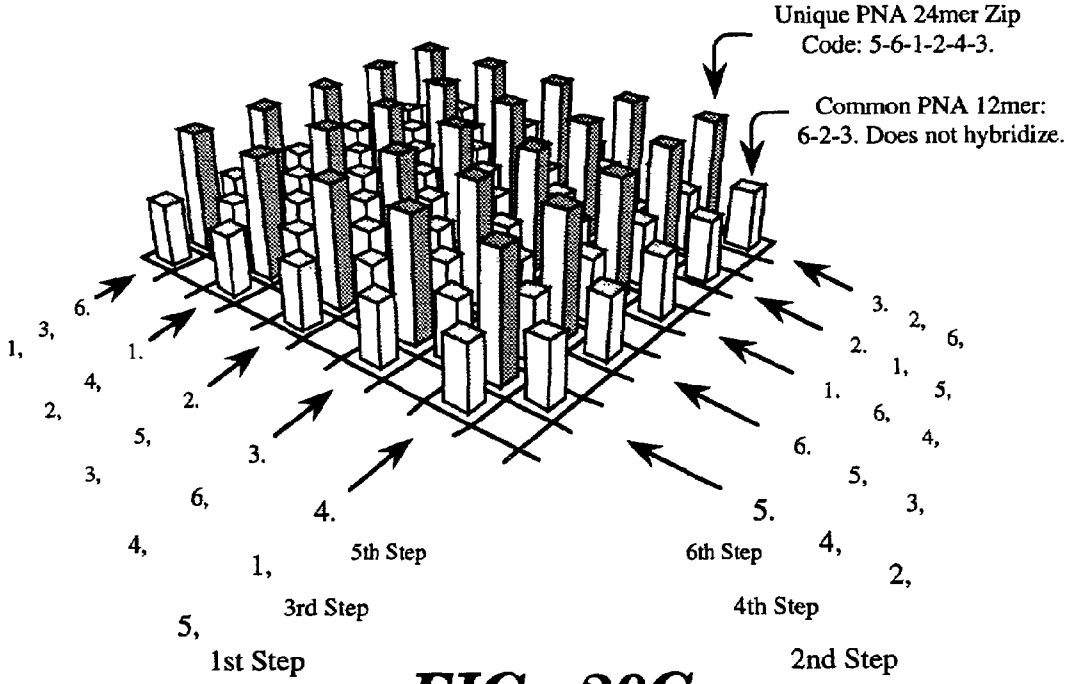


FIG. 20C

FIG. 21A



FIG. 21B



FIG. 21C

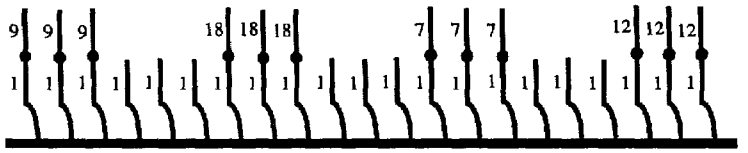


FIG. 21D

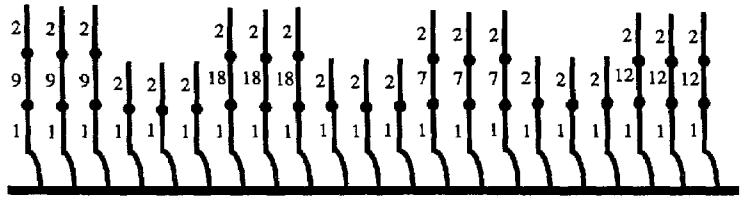


FIG. 21E

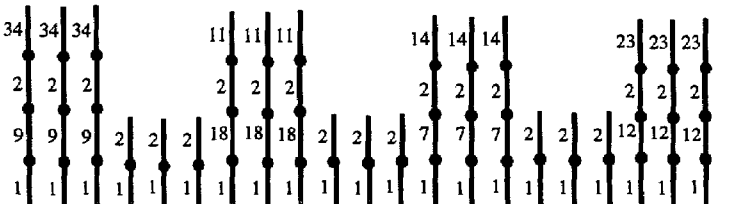
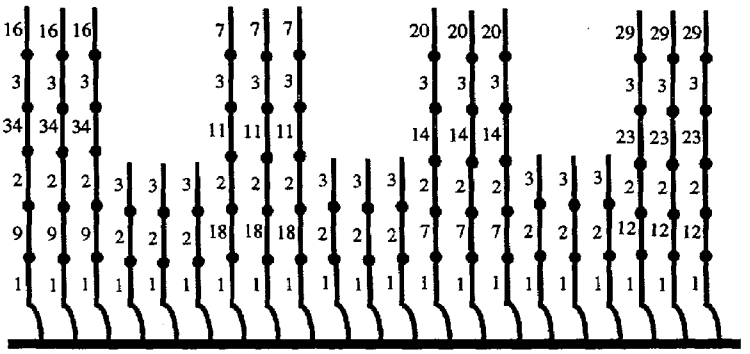


FIG. 21F



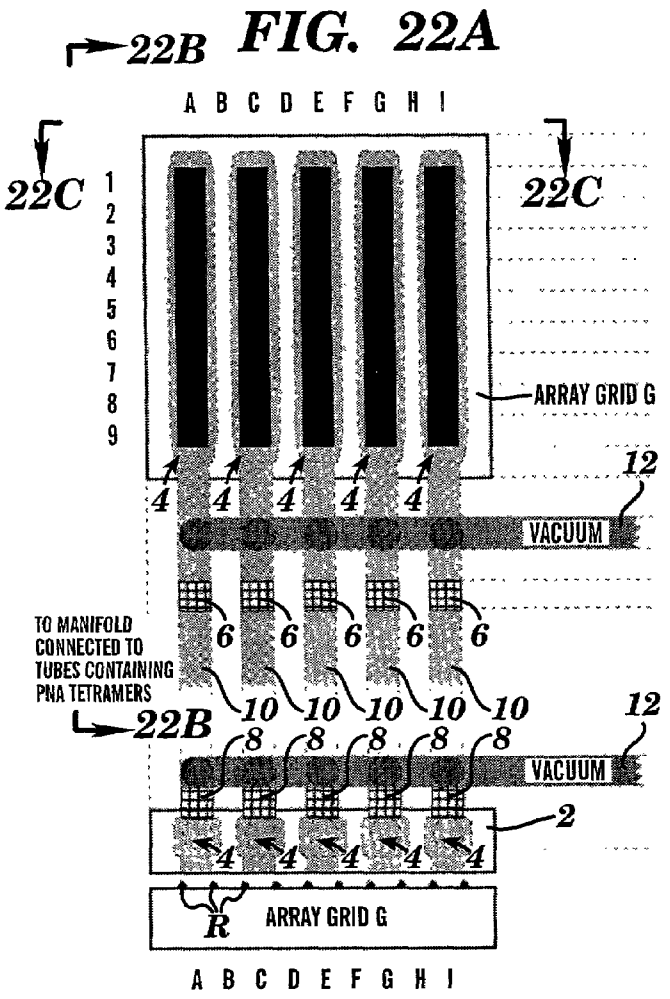


FIG. 22A

FIG. 22C

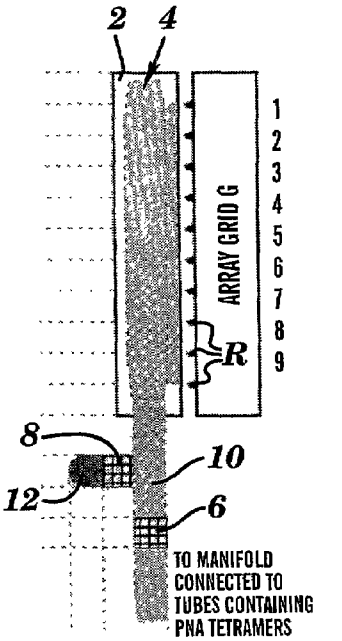


FIG. 22B

FIG. 23A

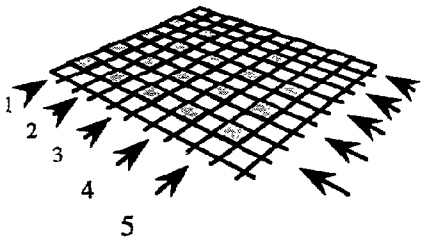
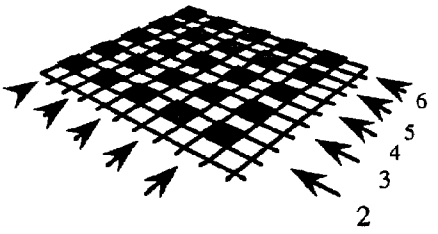


FIG. 23B



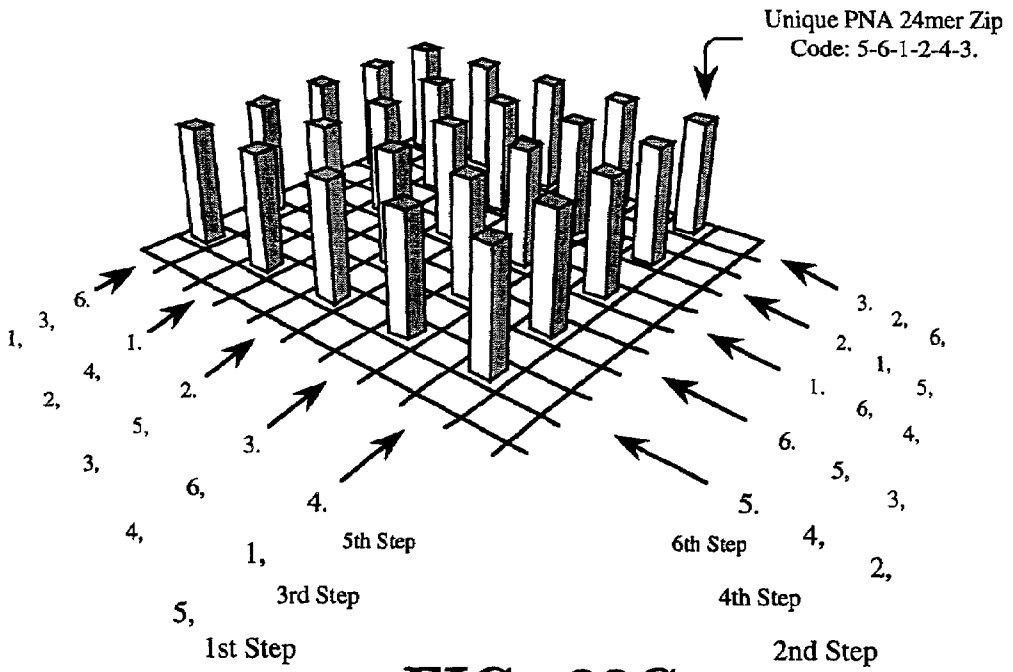


FIG. 23C

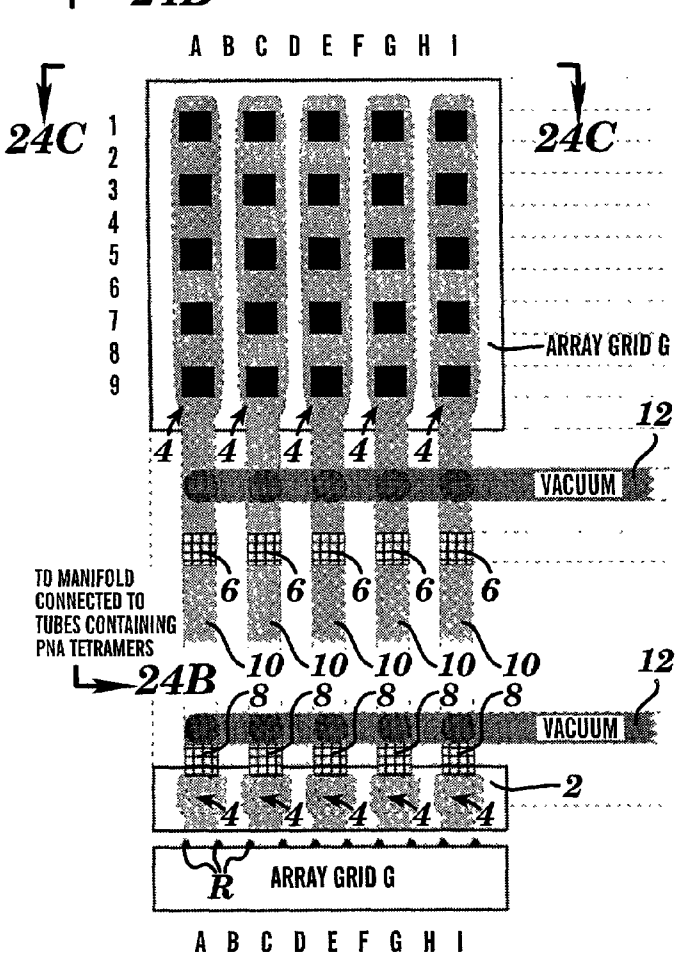


FIG. 24A

FIG. 24C

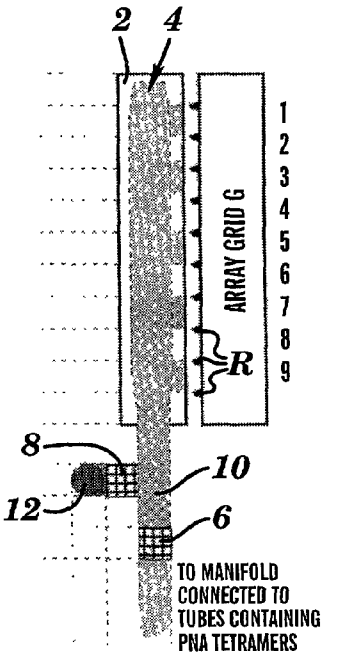


FIG. 24B

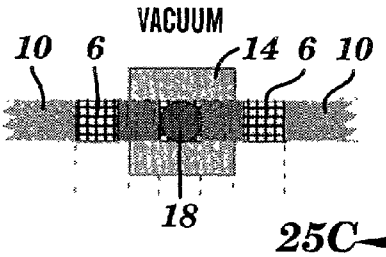


FIG. 25B

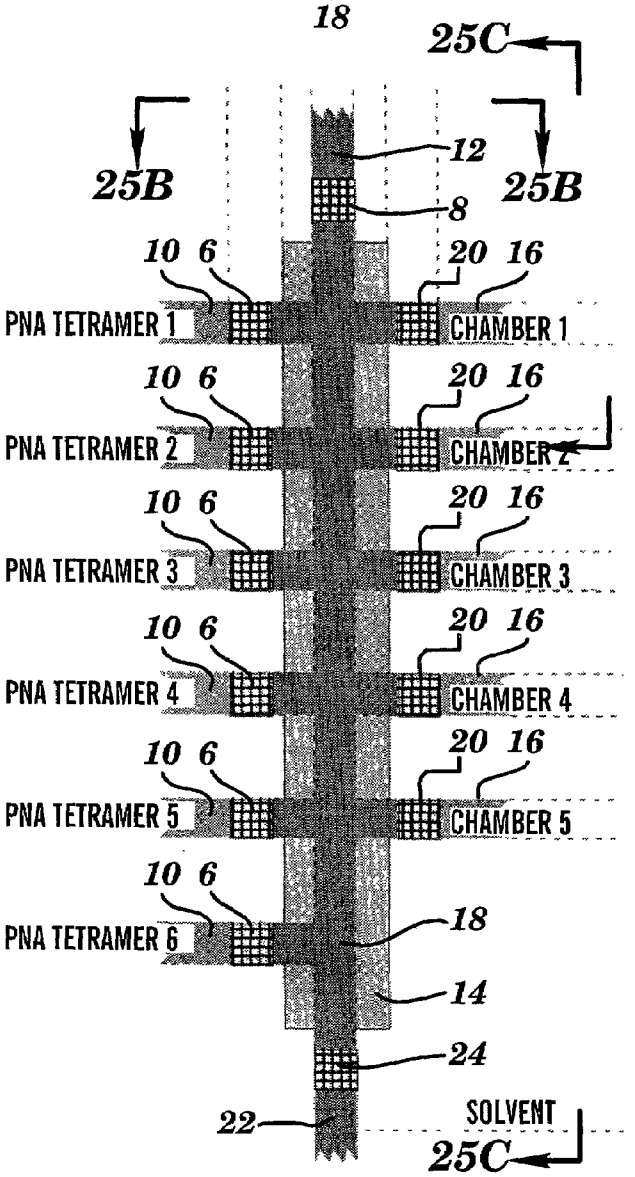


FIG. 25A

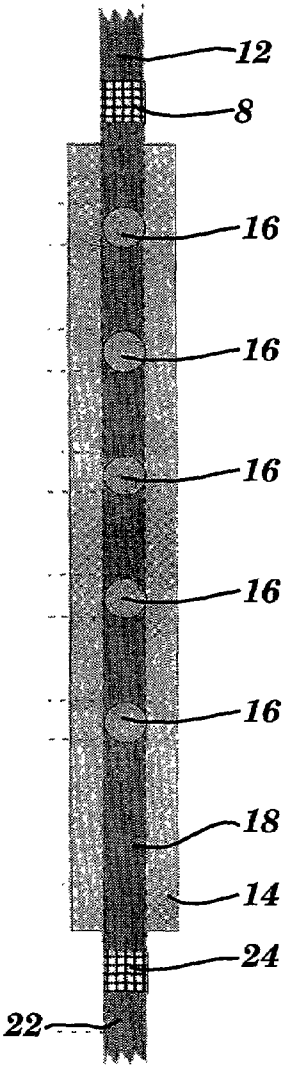


FIG. 25C

6 INPUTS AND 5 OUTPUTS

FIG. 26A

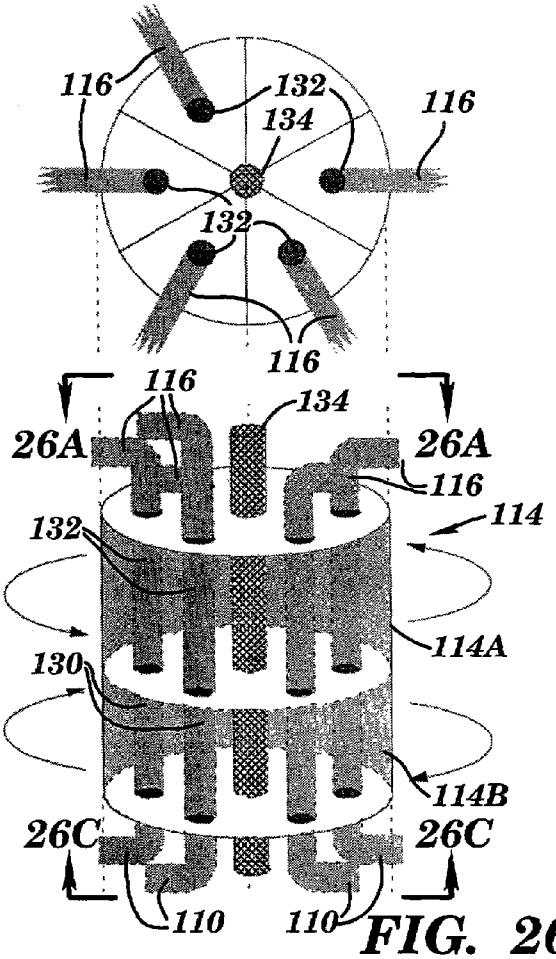


FIG. 26B

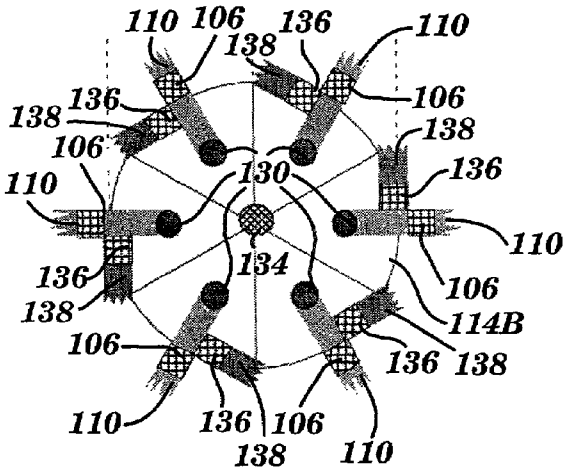


FIG. 26C

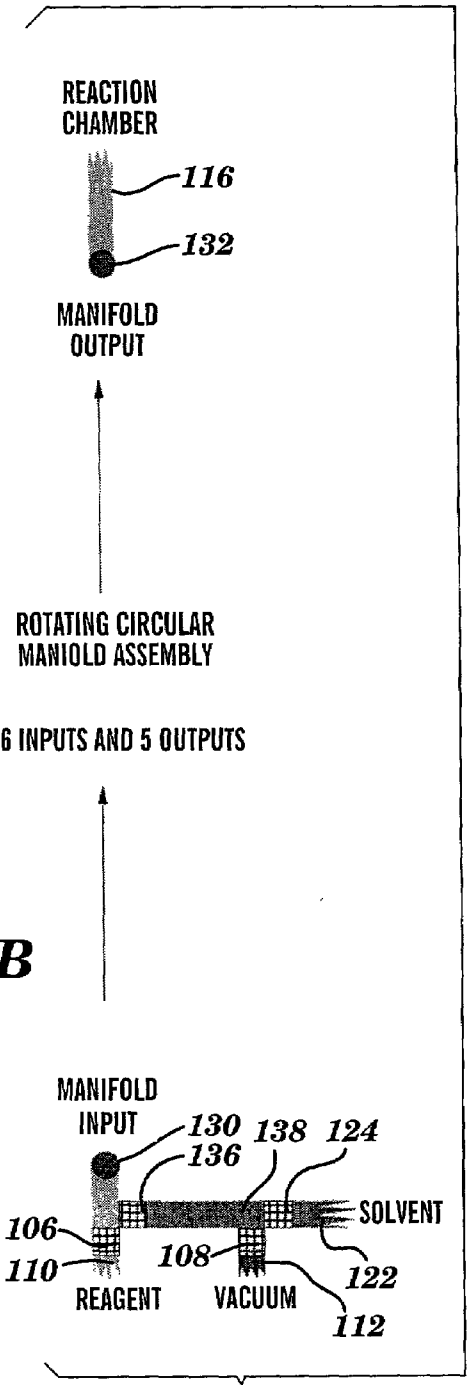


FIG. 26D

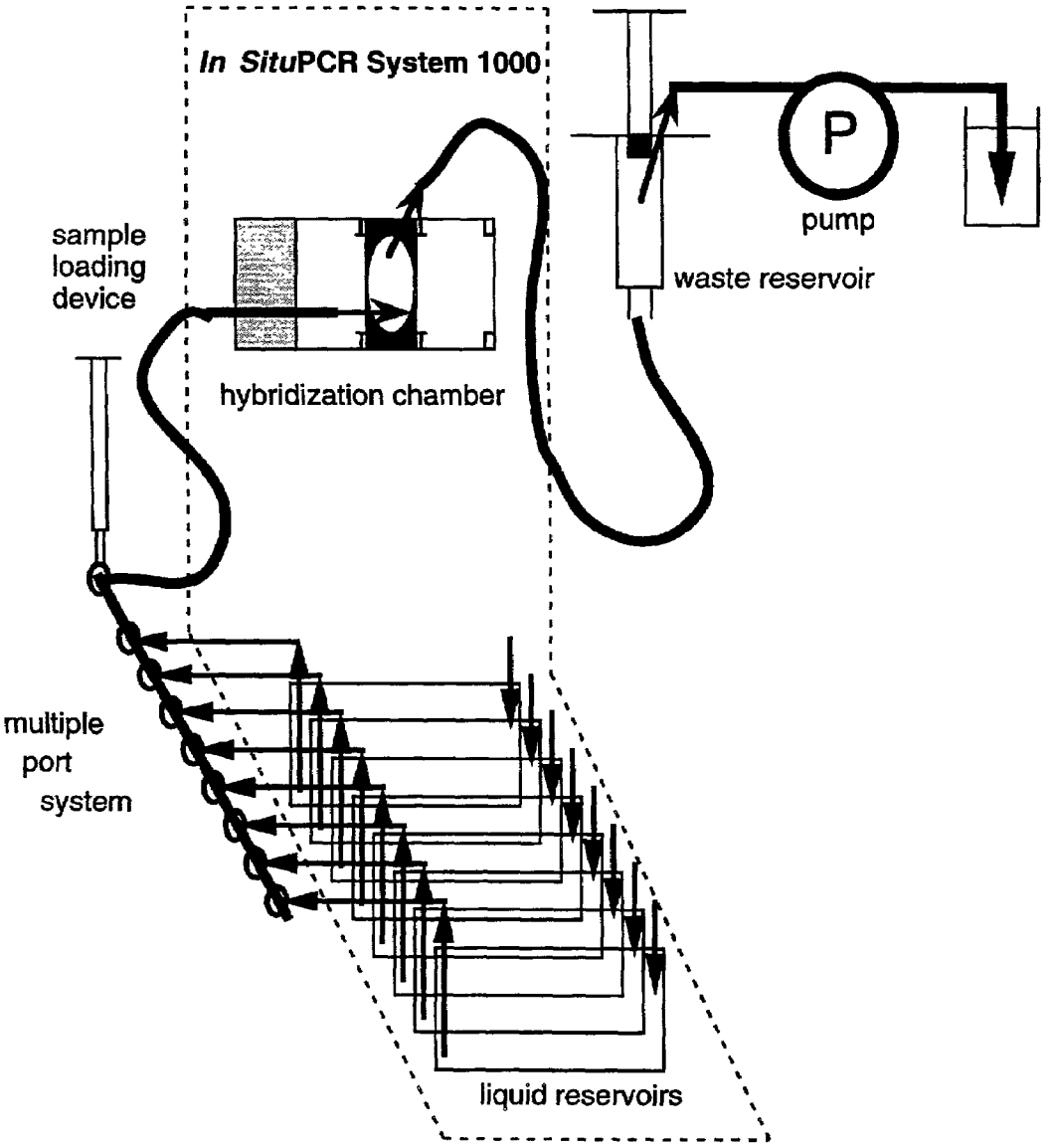

In SituPCR System 1000
P
pump
waste reservoir
sample loading device
hybridization chamber
multiple port system
liquid reservoirs

FIG. 27

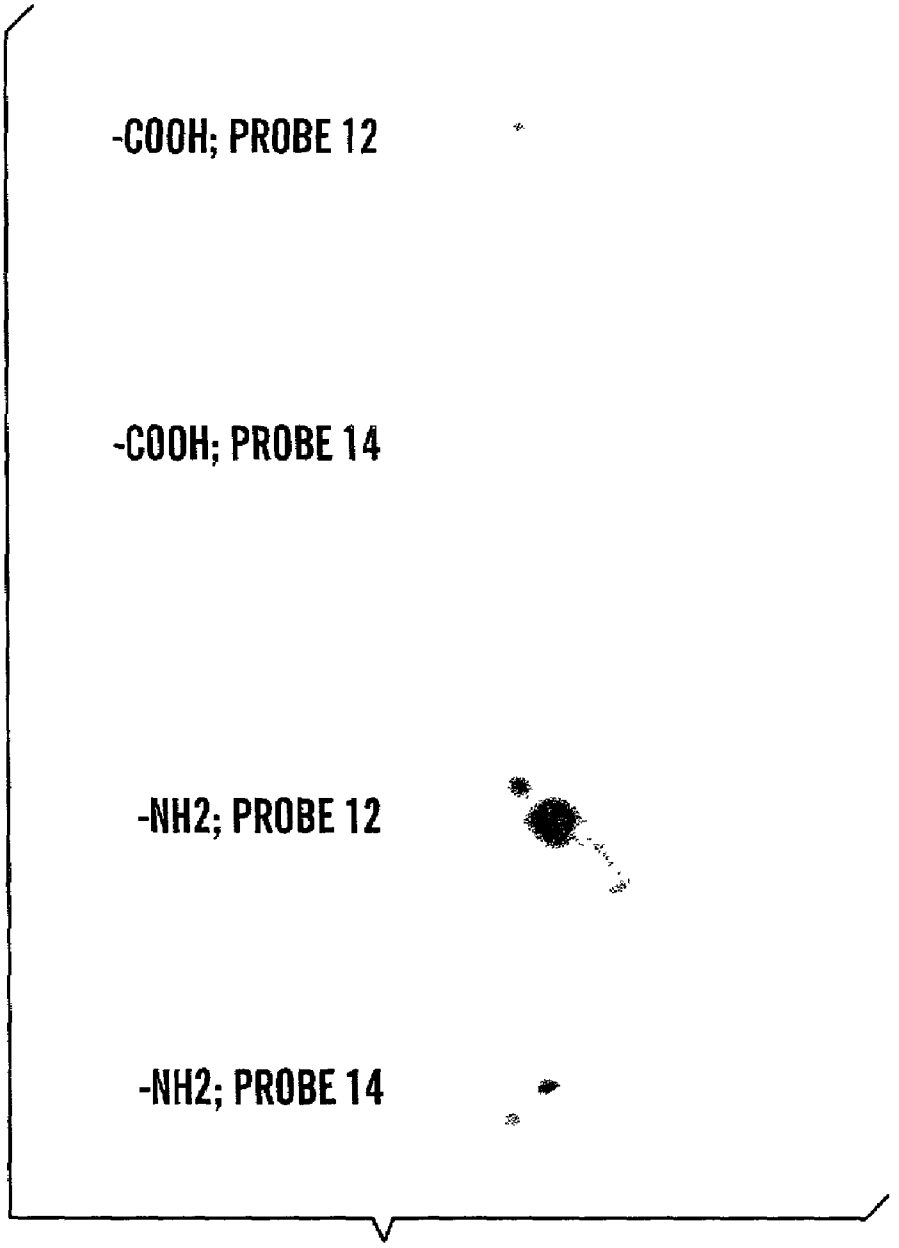


FIG. 28

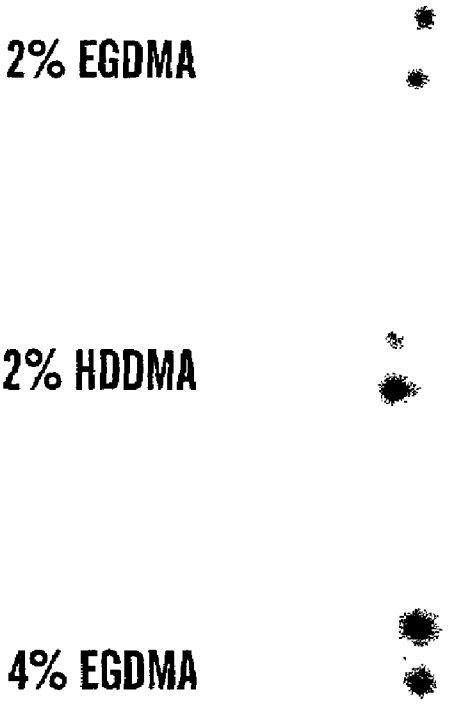


FIG. 29

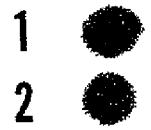


FIG. 30

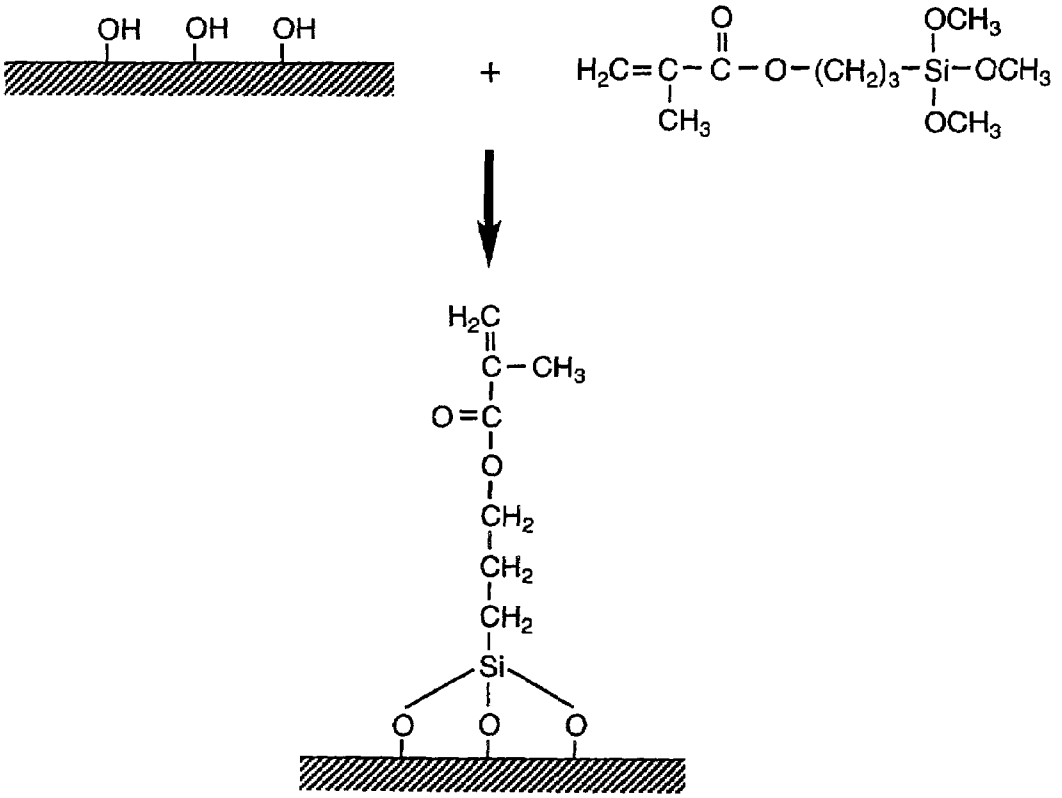

OH OH OH
+
O
OCH3
H2C=C-C-O-(CH2)3-Si-OCH3
CH3
OCH3
H2C
C-CH3
O=C
O
CH2
CH2
CH2
Si
O O O

FIG. 31

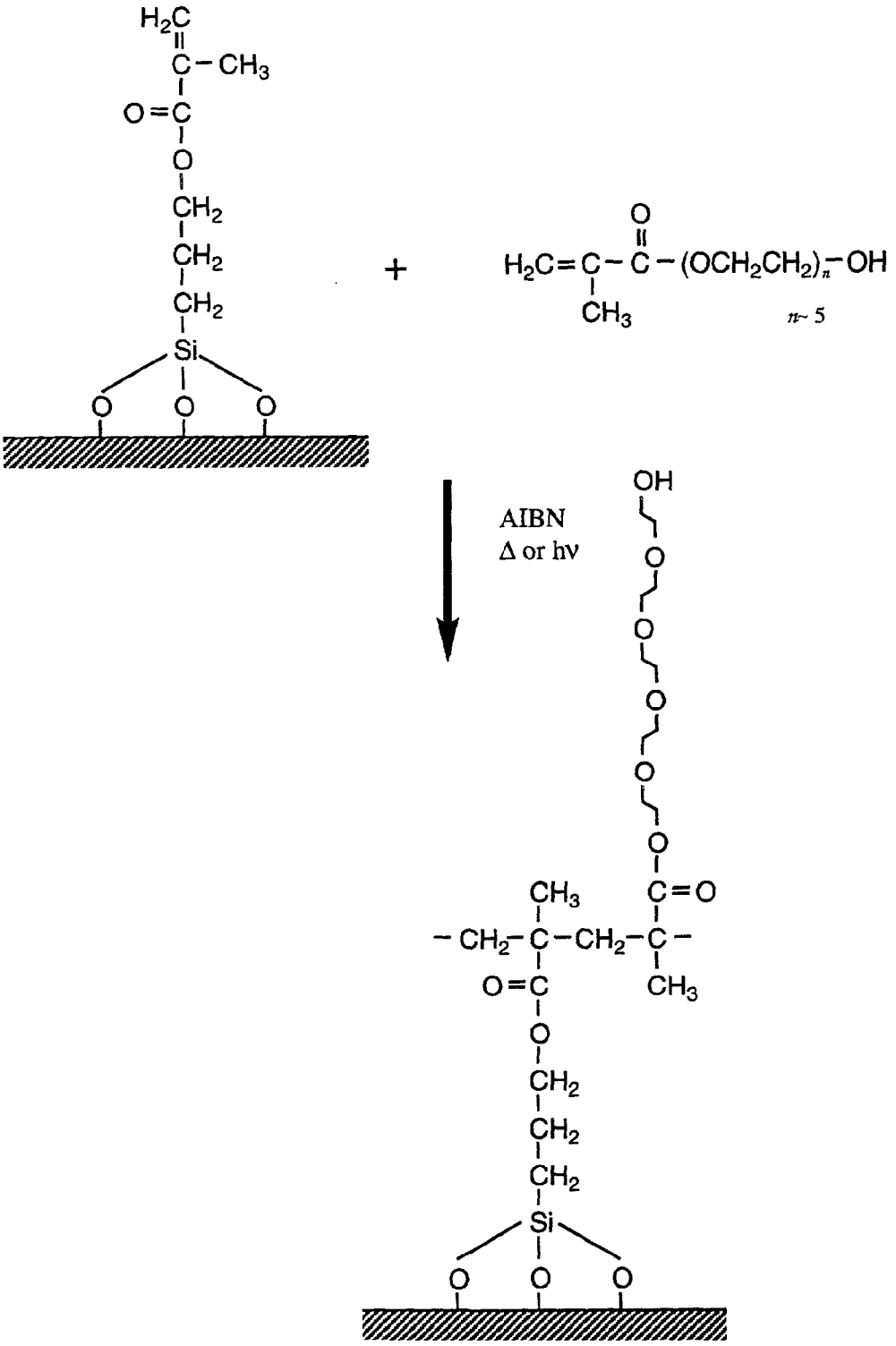


FIG. 32

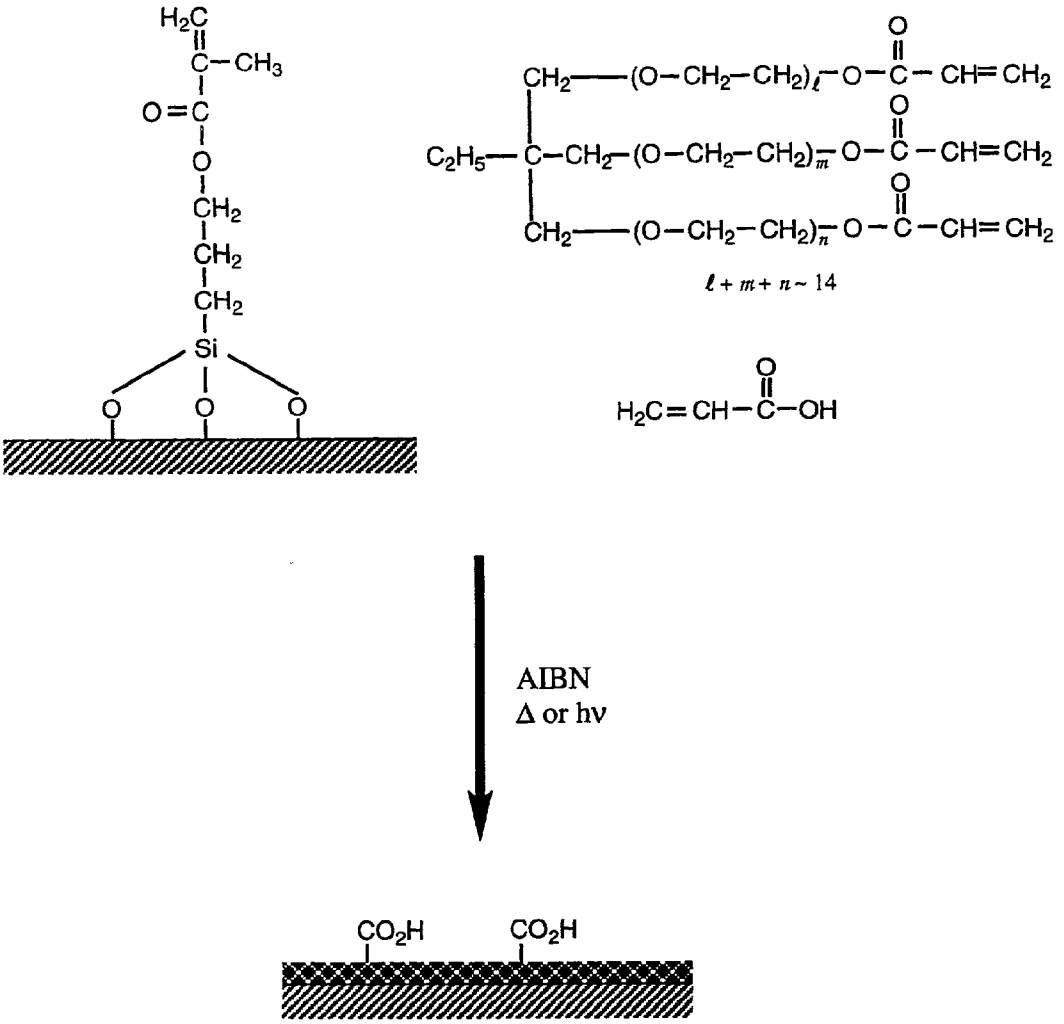


FIG. 33

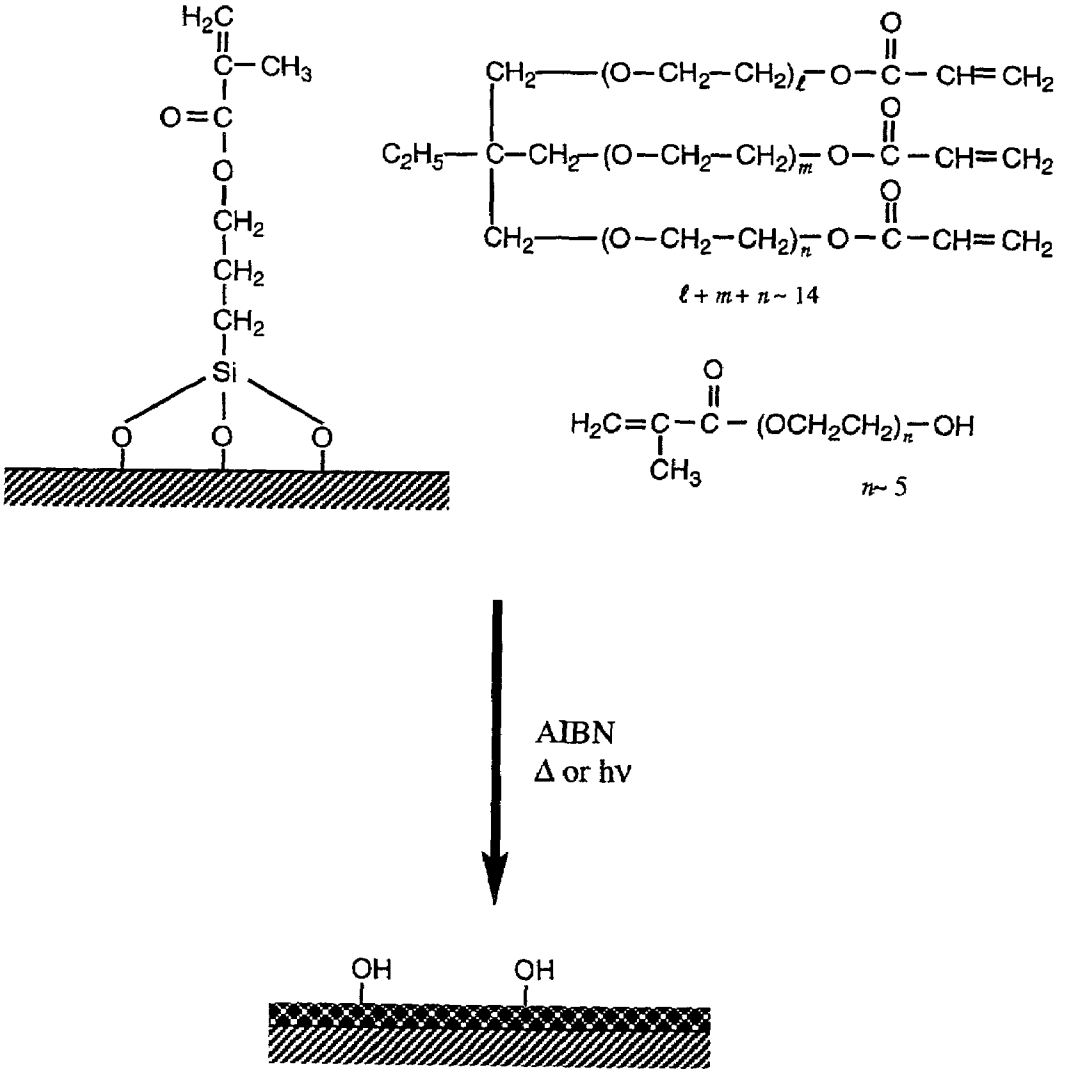


FIG. 34

DETECTION OF NUCLEIC ACID SEQUENCE DIFFERENCES USING THE LIGASE DETECTION REACTION WITH ADDRESSABLE ARRAYS

This application is a divisional of U.S. Patent Application Ser. No. 08/794,851, filed Feb. 4, 1997, now U.S. Pat. No. 6,852,487, which claims the benefit of U.S. Provisional Patent Application Ser. No. 60/011,359, filed Feb. 9, 1996.

This invention was developed with government funding under National Institutes of Health Grant Nos. GM-41337-06, GM-43552-05, GM-42722-07, and GM-51628-02. The U.S. Government may have certain rights.

FIELD OF THE INVENTION

The present invention relates to the detection of nucleic acid sequence differences in nucleic acids using a ligation phase, a capture phase, and a detection phase. The ligation phase utilizes a ligation detection reaction between one oligonucleotide probe which has a target sequence-specific portion and an addressable array-specific portion and a second oligonucleotide probe having a target sequence-specific portion and a detectable label. The capture phase involves hybridizing the ligated oligonucleotide probes to a solid support with an array of immobilized capture oligonucleotides at least some of which are complementary to the addressable array-specific portion. The labels of ligated oligonucleotide probes hybridized to the solid support are detected during the detection phase.

BACKGROUND OF THE INVENTION

Detection of Sequence Differences

Large-scale multiplex analysis of highly polymorphic loci is needed for practical identification of individuals, e.g., for paternity testing and in forensic science (Reynolds et al., *Anal. Chem.,* 63:2–15 (1991)), for organ-transplant donor-recipient matching (Buyse et al., *Tissue Antigens,* 41:1–14 (1993) and Gyllensten et al., *PCR Meth. Appl,* 1:91–98 (1991)), for genetic disease diagnosis, prognosis, and prenatal counseling (Chamberlain et al., *Nucleic Acids Res.,* 16:11141–11156 (1988) and L. C. Tsui, *Human Mutat.,* 1:197–203 (1992)), and the study of oncogenic mutations (Hollstein et al., *Science,* 253:49–53 (1991)). In addition, the cost-effectiveness of infectious disease diagnosis by nucleic acid analysis varies directly with the multiplex scale in panel testing. Many of these applications depend on the discrimination of single-base differences at a multiplicity of sometimes closely space loci.

A variety of DNA hybridization techniques are available for detecting the presence of one or more selected polynucleotide sequences in a sample containing a large number of sequence regions. In a simple method, which relies on fragment capture and labeling, a fragment containing a selected sequence is captured by hybridization to an immobilized probe. The captured fragment can be labeled by hybridization to a second probe which contains a detectable reporter moiety.

Another widely used method is Southern blotting. In this method, a mixture of DNA fragments in a sample are fractionated by gel electrophoresis, then fixed on a nitrocellulose filter. By reacting the filter with one or more labeled probes under hybridization conditions, the presence of bands containing the probe sequence can be identified. The method is especially useful for identifying fragments in a restriction-enzyme DNA digest which contain a given probe sequence, and for analyzing restriction-fragment length polymorphisms ("RFLPs").

Another approach to detecting the presence of a given sequence or sequences in a polynucleotide sample involves selective amplification of the sequence(s) by polymerase chain reaction. U.S. Pat. No. 4,683,202 to Mullis, et al. and R. K. Saiki, et al., *Science* 230:1350 (1985). In this method, primers complementary to opposite end portions of the selected sequence(s) are used to promote, in conjunction with thermal cycling, successive rounds of primer-initiated replication. The amplified sequence may be readily identified by a variety of techniques. This approach is particularly useful for detecting the presence of low-copy sequences in a polynucleotide-containing sample, e.g., for detecting pathogen sequences in a body-fluid sample.

More recently, methods of identifying known target sequences by probe ligation methods have been reported. U.S. Pat. No. 4,883,750 to N. M. Whiteley, et al., D. Y. Wu, et al., *Genomics* 4:560 (1989), U. Landegren, et al., *Science* 241:1077 (1988), and E. Winn-Deen, et al., *Clin. Chem.* 37:1522 (1991). In one approach, known as oligonucleotide ligation assay ("OLA"), two probes or probe elements which span a target region of interest are hybridized with the target region. Where the probe elements match (basepair with) adjacent target bases at the confronting ends of the probe elements, the two elements can be joined by ligation, e.g., by treatment with ligase. The ligated probe element is then assayed, evidencing the presence of the target sequence.

In a modification of this approach, the ligated probe elements act as a template for a pair of complementary probe elements. With continued cycles of denaturation, hybridization, and ligation in the presence of the two complementary pairs of probe elements, the target sequence is amplified geometrically, i.e., exponentially allowing very small amounts of target sequence to be detected and/or amplified. This approach is referred to as ligase chain reaction ("LCR"). F. Barany, "Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase," *Proc. Nat'l Acad. Sci. USA,* 88:189–93 (1991) and F. Barany, "The Ligase Chain Reaction (LCR) in a PCR World," *PCR Methods and Applications,* 1:5–16 (1991).

Another scheme for multiplex detection of nucleic acid sequence differences is disclosed in U.S. Pat. No. 5,470,705 to Grossman et. al. where sequence-specific probes, having a detectable label and a distinctive ratio of charge/translational frictional drag, can be hybridized to a target and ligated together. This technique was used in Grossman, et. al., "High-density Multiplex Detection of Nucleic Acid Sequences: Oligonucleotide Ligation Assay and Sequence-coded Separation," *Nucl. Acids Res.* 22(21):4527–34 (1994) for the large scale multiplex analysis of the cystic fibrosis transmembrane regulator gene.

Jou, et. al., "Deletion Detection in Dystrophin Gene by Multiplex Gap Ligase Chain Reaction and Immunochromatographic Strip Technology," *Human Mutation* 5:8693 (1995) relates to the use of a so called "gap ligase chain reaction" process to amplify simultaneously selected regions of multiple exons with the amplified products being read on an immunochromatographic strip having antibodies specific to the different haptens on the probes for each exon.

There is a growing need, e.g., in the field of genetic screening, for methods useful in detecting the presence or absence of each of a large number of sequences in a target polynucleotide. For example, as many as 400 different mutations have been associated with cystic fibrosis. In screening for genetic predisposition to this disease, it is

optimal to test all of the possible different gene sequence mutations in the subject's genomic DNA, in order to make a positive identification of "cystic fibrosis". It would be ideal to test for the presence or absence of all of the possible mutation sites in a single assay. However, the prior-art methods described above are not readily adaptable for use in detecting multiple selected sequences in a convenient, automated single-assay format.

Solid-phase hybridization assays require multiple liquid-handling steps, and some incubation and wash temperatures must be carefully controlled to keep the stringency needed for single-nucleotide mismatch discrimination. Multiplexing of this approach has proven difficult as optimal hybridization conditions vary greatly among probe sequences.

Allele-specific PCR products generally have the same size, and a given amplification tube is scored by the presence or absence of the product band in the gel lane associated with each reaction tube. Gibbs et al., *Nucleic Acids Res.,* 17:2437–2448 (1989). This approach requires splitting the test sample among multiple reaction tubes with different primer combinations, multiplying assay cost. PCR has also discriminated alleles by attaching different fluorescent dyes to competing allelic primers in a single reaction tube (F. F. Chehab, et al., *Proc. Natl. Acad. Sci. USA,* 86:9178–9182 (1989)), but this route to multiplex analysis is limited in scale by the relatively few dyes which can be spectrally resolved in an economical manner with existing instrumentation and dye chemistry. The incorporation of bases modified with bulky side chains can be used to differentiate allelic PCR products by their electrophoretic mobility, but this method is limited by the successful incorporation of these modified bases by polymerase, and by the ability of electrophoresis to resolve relatively large PCR products which differ in size by only one of these groups. Livak et al., *Nucleic Acids Res.,* 20:4831–4837 (1989). Each PCR product is used to look for only a single mutation, making multiplexing difficult.

Ligation of allele-specific probes generally has used solid-phase capture (U. Landegren et al., *Science,* 241:1077–1080 (1988); Nickerson et al., *Proc. Natl. Acad. Sci. USA,* 87:8923–8927 (1990)) or size-dependent separation (D. Y. Wu, et al., *Genomics,* 4:560–569 (1989) and F. Barany, *Proc. Natl. Acad. Sci.,* 88:189–193 (1991)) to resolve the allelic signals, the latter method being limited in multiplex scale by the narrow size range of ligation probes. The gap ligase chain reaction process requires an additional step—polymerase extension. The use of probes with distinctive ratios of charge/translational frictional drag technique to a more complex multiplex will either require longer electrophoresis times or the use of an alternate form of detection.

The need thus remains for a rapid single assay format to detect the presence or absence of multiple selected sequences in a polynucleotide sample.

Use of Oligonucleotide Arrays for Nucleic Acid Analysis

Ordered arrays of oligonucleotides immobilized on a solid support have been proposed for sequencing, sorting, isolating, and manipulating DNA. It has been recognized that hybridization of a cloned single-stranded DNA molecule to all possible oligonucleotide probes of a given length can theoretically identify the corresponding complementary DNA segments present in the molecule. In such an array, each oligonucleotide probe is immobilized on a solid support at a different predetermined position. All the oligonucleotide segments in a DNA molecule can be surveyed with such an array.

One example of a procedure for sequencing DNA molecules using arrays of oligonucleotides is disclosed in U.S. Pat. No. 5,202,231 to Drmanac, et. al. *This involves application of target DNA to a solid support to which a plurality of oligonucleotides are attached. Sequences are read by hybridization of segments of the target DNA to the oligonucleotides and assembly of overlapping segments of hybridized oligonucleotides. The array utilizes all possible oligonucleotides of a certain length between* 11 and 20 nucleotides, but there is little information about how this array is constructed. See also A. B. Chetverin, et. al., "Sequencing of Pools of Nucleic Acids on Oligonucleotide Arrays," *BioSystems* 30: 215–31 (1993); WO 92/16655 to Khrapko et. al.; Kuznetsova, et. al., "DNA Sequencing by Hybridization with Oligonucleotides Immobilized in Gel. Chemical Ligation as a Method of Expanding the Prospects for the Method," *Mol. Biol.* 28(20): 290–99(1994); M. A. Livits, et. al., "Dissociation of Duplexes Formed by Hybridization of DNA with Gel-Immobilized Oligonucleotides," *J. Biomolec. Struct. & Dynam.* 11(4): 783–812 (1994).

WO 89/10977 to Southern discloses the use of a support carrying an array of oligonucleotides capable of undergoing a hybridization reaction for use in analyzing a nucleic acid sample for known point mutations, genomic fingerprinting, linkage analysis, and sequence determination. The matrix is formed by laying nucleotide bases in a selected pattern on the support. This reference indicates that a hydroxyl linker group can be applied to the support with the oligonucleotides being assembled by a pen plotter or by masking.

WO 94/11530 to Cantor also relates to the use of an oligonucleotide array to carry out a process of sequencing by hybridization. The oligonucleotides are duplexes having overhanging ends to which target nucleic acids bind and are then ligated to the non-overhanging portion of the duplex. The array is constructed by using streptavidin-coated filter paper which captures biotinylated oligonucleotides assembled before attachment.

WO 93/17126 to Chetverin uses sectioned, binary oligonucleotide arrays to sort and survey nucleic acids. These arrays have a constant nucleotide sequence attached to an adjacent variable nucleotide sequence, both bound to a solid support by a covalent linking moiety. The constant nucleotide sequence has a priming region to permit amplification by PCR of hybridized strands. Sorting is then carried out by hybridization to the variable region. Sequencing, isolating, sorting, and manipulating fragmented nucleic acids on these binary arrays are also disclosed. In one embodiment with enhanced sensitivity, the immobilized oligonucleotide has a shorter complementary region hybridized to it, leaving part of the oligonucleotide uncovered. The array is then subjected to hybridization conditions so that a complementary nucleic acid anneals to the immobilized oligonucleotide. DNA ligase is then used to join the shorter complementary region and the complementary nucleic acid on the array. There is little disclosure of how to prepare the arrays of oligonucleotides.

WO 92/10588 to Fodor et. al., discloses a process for sequencing, fingerprinting, and mapping nucleic acids by hybridization to an array of oligonucleotides. The array of oligonucleotides is prepared by a very large scale immobilized polymer synthesis which permits the synthesis of large, different oligonucleotides. In this procedure, the substrate surface is functionalized and provided with a linker group by which oligonucleotides are assembled on the substrate. The regions where oligonucleotides are attached have protective groups (on the substrate or individual nucleotide subunits) which are selectively activated. Generally, this involves

imaging the array with light using a mask of varying configuration so that areas exposed are deprotected. Areas which have been deprotected undergo a chemical reaction with a protected nucleotide to extend the oligonucleotide sequence where imaged. A binary masking strategy can be used to build two or more arrays at a given time. Detection involves positional localization of the region where hybridization has taken place. See also U.S. Pat. Nos. 5,324,633 and 5,424,186 to Fodor et. al., U.S. Pat. Nos. 5,143,854 and 5,405,783 to Pirrung, et. al., WO 90/15070 to Pirrung, et. al., A. C. Pease, et. al., "Light-generated Oligonucleotide Arrays for Rapid DNA Sequence Analysis", *Proc. Natl. Acad. Sci USA* 91: 5022–26 (1994). K. L. Beattie, et. al., "Advances in Genosensor Research," *Clin. Chem.* 41(5): 700–09 (1995) discloses attachment of previously assembled oligonucleotide probes to a solid support.

There are many drawbacks to the procedures for sequencing by hybridization to such arrays. Firstly, a very large number of oligonucleotides must be synthesized. Secondly, there is poor discrimination between correctly hybridized, properly matched duplexes and those which are mismatched. Finally, certain oligonucleotides will be difficult to hybridize to under standard conditions, with such oligonucleotides being capable of identification only through extensive hybridization studies.

The present invention is directed toward overcoming these deficiencies in the art.

SUMMARY OF THE INVENTION

The present invention relates to a method for identifying one or more of a plurality of sequences differing by one or more single base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. The method includes a ligation phase, a capture phase, and a detection phase.

The ligation phase requires providing a sample potentially containing one or more nucleotide sequences with a plurality of sequence differences. A plurality of oligonucleotide sets are utilized in this phase. Each set includes a first oligonucleotide probe, having a target-specific portion and an addressable array-specific portion, and a second oligonucleotide probe, having a target-specific portion and a detectable reporter label. The first and second oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target nucleotide sequence. However, the first and second oligonucleotide probes have a mismatch which interferes with such ligation when hybridized to another nucleotide sequence present in the sample. A ligase is also utilized. The sample, the plurality of oligonucleotide probe sets, and the ligase are blended to form a mixture. The mixture is subjected to one or more ligase detection reaction cycles comprising a denaturation treatment and a hybridization treatment. The denaturation treatment involves separating any hybridized oligonucleotides from the target nucleotide sequences. The hybridization treatment involves hybridizing the oligonucleotide probe sets at adjacent positions in a base-specific manner to their respective target nucleotide sequences, if present in the sample, and ligating them to one another to form a ligated product sequence containing (a) the addressable array-specific portion, (b) the target-specific portions connected together, and (c) the detectable reporter label. The oligonucleotide probe sets may hybridize to nucleotide sequences in the sample other than their respective target nucleotide sequences but do not ligate together due to a presence of one or more mismatches and individually separate during denaturation treatment.

The next phase of the process is the capture phase. This phase involves providing a solid support with capture oligonucleotides immobilized at particular sites. The capture oligonucleotides are complementary to the addressable array-specific portions. The mixture, after being subjected to the ligation phase, is contacted with the solid support under conditions effective to hybridize the addressable array-specific portions to the capture oligonucleotides in a base-specific manner. As a result, the addressable array-specific portions are captured on the solid support at the site with the complementary capture oligonucleotides.

After the capture phase is the detection phase. During this portion of the process, the reporter labels of the ligated product sequences are captured on the solid support at particular sites. When the presence of the reporter label bound to the solid support is detected, the respective presence of one or more nucleotide sequences in the sample is indicated.

The present invention also relates to a kit for carrying out the method of the present invention which includes the ligase, the plurality of oligonucleotide sets, and the solid support with immobilized capture oligonucleotides.

Another aspect of the present invention relates to a method of forming an array of oligonucleotides on a solid support. This method involves providing a solid support having an array of positions each suitable for attachment of an oligonucleotide. A linker or surface (which can be non-hydrolyzable), suitable for coupling an oligonucleotide to the solid support at each of the array positions, is attached to the solid support. An array of oligonucleotides on a solid support is formed by a series of cycles of activating selected array positions for attachment of multimer nucleotides and attaching multimer nucleotides at the activated array positions.

Yet another aspect of the present invention relates to an array of oligonucleotides on a solid support per se. The solid support has an array of positions each suitable for attachment of an oligonucleotide. A linker or support (which can be non-hydrolyzable), suitable for coupling an oligonucleotide to the solid support, is attached to the solid support at each of the array positions. An array of oligonucleotides are placed on a solid support with at least some of the array positions being occupied by oligonucleotides having greater than sixteen nucleotides.

The present invention contains a number of advantages over prior art systems, particularly, its ability to carry out multiplex analyses of complex genetic systems. As a result, a large number of nucleotide sequence differences in a sample can be detected at one time. The present invention is useful for detection of, for example, cancer mutations, inherited (germline) mutations, and infectious diseases. This technology can also be utilized in conjunction with environmental monitoring, forensics, and the food industry.

In addition, the present invention provides quantitative detection of mutations in a high background of normal sequences, allows detection of closely-clustered mutations, permits detection using addressable arrays, and is amenable to automation. By combining the sensitivity of PCR with the specificity of LDR, common difficulties encountered in allele-specific PCR, such as false-positive signal generation, primer interference during multiplexing, limitations in obtaining quantitative data, and suitability for automation, have been obviated. In addition, by relying on the specificity of LDR to distinguish single-base mutations, the major inherent problem of oligonucleotide probe arrays (i.e. their

inability to distinguish single-base changes at all positions in heterozygous samples) has been overcome. PCR/LDR addresses the current needs in cancer detection; to quantify mutations which may serve as clonal markers and to detect minimal residual disease and micrometastases.

In carrying out analyses of different samples, the solid support containing the array can be reused. This reduces the quantity of solid supports which need to be manufactured and lowers the cost of analyzing each sample.

The present invention also affords great flexibility in the synthesis of oligonucleotides and their attachment to solid supports. Oligonucleotides can be synthesized off of the solid support and then attached to unique surfaces on the support. This technique can be used to attach full length oligonucleotides or peptide nucleotide analogues ("PNA") to the solid support. Alternatively, shorter nucleotide or analogue segments (dimer, trimer, tetramer, etc.) can be employed in a segment condensation or block synthesis approach to full length oligomers on the solid support.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a flow diagram depicting polymerase chain reaction ("PCR")/ligase detection reaction ("LDR") processes, according to the prior art and the present invention, for detection of germline mutations, such as point mutations.

FIG. **2** is a flow diagram depicting PCR/LDR processes, according to the prior art and the present invention, for detection of cancer-associated mutations.

FIG. **3** is a schematic diagram depicting a PCR/LDR process, according to the present invention, using addresses on the allele-specific probes for detecting homo- or heterozygosity at two polymorphisms (i.e. allele differences) on the same gene.

FIG. **4** is a schematic diagram depicting a PCR/LDR process, according to the present invention, using addresses on the allele-specific probes which distinguishes all possible bases at a given site.

FIG. **5** is a schematic diagram depicting a PCR/LDR process, according to the present invention, using addresses on the allele-specific probes for detecting the presence of any possible base at two nearby sites.

FIG. **6** is a schematic diagram of a PCR/LDR process, according to the present invention, using addresses on the allele-specific probes distinguishing insertions and deletions.

FIG. **7** is a schematic diagram of a PCR/LDR process, in accordance with the present invention, using addresses on the allele-specific probes to detect a low abundance mutation (within a codon) in the presence of an excess of normal sequence.

FIG. **8** is a schematic diagram of a PCR/LDR process, in accordance with the present invention, where the address is placed on the common probe and the allele differences are distinguished by different fluorescent signals F**1**, F**2**, F**3**, and F**4**.

FIG. **9** is a schematic diagram of a PCR/LDR process, in accordance with the present invention, where both adjacent and nearby alleles are detected.

FIG. **10** is a schematic diagram of a PCR/LDR process, in accordance with the present invention, where all possible single-base mutations for a single codon are detected.

FIG. **11** shows the chemical reactions for covalent modifications, grafting, and oligomer attachments to solid supports.

FIGS. **12**A–C show proposed chemistries for covalent attachment of oligonucleotides to solid supports.

FIGS. **13**A–C show two alternative formats for oligonucleotide probe capture. In FIG. **13**B, the addressable array-specific portions are on the allele-specific probe. Alleles are distinguished by capture of fluorescent signals on addresses Z**1** and Z**2**, respectively. In FIG. **13**C, the addressable array-specific portions are on the common probe and alleles are distinguished by capture of fluorescent signals F**1** and F**2**, which correspond to the two alleles, respectively.

FIGS. **14**A–E depict a protocol for constructing an 8×8 array of oligomers by spotting full-length, individual 24 mer oligomers at various sites on a solid support.

FIGS. **15**A–E are perspective views of the 8×8 array construction protocol of FIGS. **14**A–E.

FIGS. **16**A–C are views of an apparatus used to spot full-length, individual 24 mer oligomers on a solid support in accordance with FIGS. **14**A–E to **15** A–E.

FIG. **17** shows a design in accordance with the present invention using 36 tetramers differing by at least 2 bases, which can be used to create a series of unique 24-mers.

FIGS. **18**A–G are schematic diagrams showing addition of PNA tetramers to generate a 5×5 array of unique 25 mer addresses.

FIGS. **19**A–E depict a protocol for constructing an 8×8 array of 24-mers by sequentially coupling 6 tetramers.

FIGS. **20**A–C are perspective views of the 8×8 array construction protocol of FIGS. **19**B–C.

FIGS. **21**A–F show a schematic cross-sectional view of the synthesis of an addressable array, in accordance with FIGS. **19**B–C.

FIGS. **22**A–C are schematic views of an apparatus used to synthesize the 8×8 array of 24 mers on a solid support in accordance with FIGS. **19**B–C, **20**A–C, and **21**A–F.

FIGS. **23**A–C are perspective views of the 8×8 array construction protocol of FIG. **19** (FIGS. **19**D–**19**E).

FIGS. **24**A–C are schematic views of an apparatus used to synthesize the 5×5 array of 24 mers on a solid support, in accordance with FIGS. **19**D–E and **23** A–C.

FIGS. **25**A–C are schematic diagrams of a valve block assembly capable of routing six input solutions to 5 output ports.

FIGS. **26**A–D are diagrams of a circular manifold capable of simultaneously channeling 6 input solutions into 5 output ports.

FIG. **27** is a schematic drawing of an assay system for carrying out the process of the present invention.

FIG. **28** shows phosphorimager data for different derivatized surfaces.

FIG. **29** shows phosphorimager data for different crosslinking conditions of the polymer matrix.

FIG. **30** shows phosphorimager data for —OH functionalized slides.

FIG. **31** shows the reaction scheme for producing a glass slide silanized with 3-methacryloyloxypropyltrimethoxysilane.

FIG. **32** shows the reaction scheme for producing polymerized poly(ethylene glycol)methacrylate on a glass slide silanized with 3-methacryloyloxypropyl-trimethoxysilane.

FIG. **33** shows the reaction scheme for producing polymerized acrylic acid and trimethylolpropane ethoxylate (14/3 EO/OH) triacrylate on a glass slide silanized with 3-methacryloyloxypropyltrimethoxysilane.

FIG. **34** shows the reaction scheme for producing polymerized poly(ethylene glycol)methacrylate and trimethylolpropane ethoxylate (14/3 EO/OH) triacrylate on a glass slide silanized with 3-methacryloyloxypropyltrimethoxysilane.

DETAILED DESCRIPTION OF THE INVENTION AND DRAWINGS

The present invention relates to a method for identifying one or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. The method includes a ligation phase, a capture phase, and a detection phase.

The ligation phase requires providing a sample potentially containing one or more nucleotide sequences with a plurality of sequence differences. A plurality of oligonucleotide sets are utilized in this phase. Each set includes a first oligonucleotide probe, having a target-specific portion and an addressable array-specific portion, and a second oligonucleotide probe, having a target-specific portion and a detectable reporter label. The first and second oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target nucleotide sequence. However, the first and second oligonucleotide probes have a mismatch which interferes with such ligation when hybridized to another nucleotide sequence present in the sample. A ligase is also utilized. The sample, the plurality of oligonucleotide probe sets, and the ligase are blended to form a mixture. The mixture is subjected to one or more ligase detection reaction cycles comprising a denaturation treatment and a hybridization treatment. The denaturation treatment involves separating any hybridized oligonucleotides from the target nucleotide sequences. The hybridization treatment involves hybridizing the oligonucleotide probe sets at adjacent positions in a base-specific manner to their respective target nucleotide sequences, if present in the sample, and ligating them to one another to form a ligated product sequence containing (a) the addressable array-specific portion, (b) the target-specific portions connected together, and (c) the detectable reporter label. The oligonucleotide probe sets may hybridize to nucleotide sequences in the sample other than their respective target nucleotide sequences but do not ligate together due to a presence of one or more mismatches and individually separate during denaturation treatment.

The next phase of the process is the capture phase. This phase involves providing a solid support with capture oligonucleotides immobilized at particular sites. The capture oligonucleotides are complementary to the addressable array-specific portions. The mixture, after being subjected to the ligation phase, is contacted with the solid support under conditions effective to hybridize the addressable array-specific portions to the capture oligonucleotides in a base-specific manner. As a result, the addressable array-specific portions are captured on the solid support at the site with the complementary capture oligonucleotides.

After the capture phase is the detection phase. During this portion of the process, the reporter labels of the ligated product sequences are captured on the solid support at particular sites. When the presence of the reporter label bound to the solid support is detected, the respective presence of one or more nucleotide sequences in the sample is indicated.

Often, a number of different single-base mutations, insertions, or deletions may occur at the same nucleotide position of the sequence of interest. The method provides for having an oligonucleotide set, where the second oligonucleotide probe is common and contains the detectable label, and the first oligonucleotide probe has different addressable array-specific portions and target-specific portions. The first oligonucleotide probe is suitable for ligation to a second adjacent oligonucleotide probe at a first ligation junction, when hybridized without mismatch, to the sequence in question. Different first adjacent oligonucleotide probes would contain different discriminating base(s) at the junction where only a hybridization without mismatch at the junction would allow for ligation. Each first adjacent oligonucleotide would contain a different addressable array-specific portion, and, thus, specific base changes would be distinguished by capture at different addresses. In this scheme, a plurality of different capture oligonucleotides are attached at different locations on the solid support for multiplex detection of additional nucleic acid sequences differing from other nucleic acids by at least a single base. Alternatively, the first oligonucleotide probe contains common addressable array-specific portions, and the second oligonucleotide probes have different detectable labels and target-specific portions.

Such arrangements permit multiplex detection of additional nucleic acid sequences differing from other nucleic acids by at least a single base. The nucleic acids sequences can be on the same or different alleles when carrying out such multiplex detection.

The present invention also relates to a kit for carrying out the method of the present invention which includes the ligase, the plurality of different oligonucleotide probe sets, and the solid support with immobilized capture oligonucleotides. Primers for preliminary amplification of the target nucleotide sequences may also be included in the kit. If amplification is by polymerase chain reaction, polymerase may also be included in the kit.

FIGS. **1** and **2** show flow diagrams of the process of the present invention compared to a prior art ligase detection reaction utilizing capillary or gel electrophoresis/fluorescent quantification. FIG. **1** relates to detection of a germline mutation detection, while FIG. **2** shows the detection of cancer.

FIG. **1** depicts the detection of a germline point mutation, such as the p53 mutations responsible for Li-Fraumeni syndrome. In step 1, after DNA sample preparation, exons **5**–**8** are PCR amplified using Taq (i.e. *Thermus aquaticus*) polymerase under hot start conditions. At the end of the reaction, Taq polymerase is degraded by heating at 100° C. for 10 min. Products are diluted 20-fold in step 2 into fresh LDR buffer containing allele-specific and common LDR probes. A tube generally contains about 100 to 200 fmoles of each primer. In step 3, the ligase detection reaction is initiated by addition of Taq ligase under hot start conditions. The LDR probes ligate to their adjacent probes only in the presence of target sequence which gives perfect complementarity at the junction site. The products may be detected in two different formats. In the first format **4***a*., used in the prior art, fluorescently-labeled LDR probes contain different length poly A or hexaethylene oxide tails. Thus, each LDR product, resulting from ligation to normal DNA with a slightly different mobility, yields a ladder of peaks. A germline mutation would generate a new peak on the electrophorogram. The size of the new peak will approximate the amount of the mutation present in the original sample; 0% for homozygous normal, 50% for heterozygous carrier, or 100% for homozygous mutant. In the second format **4***b*., in accordance with the present invention, each allele-specific probe contains e.g., 24 additional nucleotide bases on their 5' ends. These sequences are unique addressable sequences which will specifically hybridize to their complementary address sequences on an addressable array. In the LDR reaction, each allele-specific probe can ligate to its adjacent fluorescently labeled common probe in the

presence of the corresponding target sequence. Wild type and mutant alleles are captured on adjacent addresses on the array. Unreacted probes are washed away. The black dots indicate 100% signal for the wild type allele. The white dots indicate 0% signal for the mutant alleles. The shaded dots indicate the one position of germline mutation, 50% signal for each allele.

FIG. **2** depicts detection of somatic cell mutations in the p53 tumor suppressor gene but is general for all low sensitivity mutation detection. In step 1, DNA samples are prepared and exons **5**–**9** are PCR amplified as three fragments using fluorescent PCR primers. This allows for fluorescent quantification of PCR products in step 2 using capillary or gel electrophoresis. In step 3, the products are spiked with a 1/100 dilution of marker DNA (for each of the three fragments). This DNA is homologous to wild type DNA, except it contains a mutation which is not observed in cancer cells, but which may be readily detected with the appropriate LDR probes. The mixed DNA products in step 4 are diluted 20-fold into buffer containing all the LDR probes which are specific only to mutant or marker alleles. In step 5, the ligase reaction is initiated by addition of Taq ligase under hot start conditions. The LDR probes ligate to their adjacent probes only in the presence of target sequences which give perfect complementarity at the junction site. The products may be detected in the same two formats described in FIG. **1**. In the format of step 6a, which is used in the prior art, products are separated by capillary or gel electrophoresis, and fluorescent signals are quantified. Ratios of mutant peaks to marker peaks give approximate amount of cancer mutations present in the original sample divided by 100. In the format of step 6b, in accordance with the present invention, products are detected by specific hybridization to complementary sequences on an addressable array. Ratios of fluorescent signals in mutant dots to marker dots give the approximate amount of cancer mutations present in the original sample divided by 100.

The ligase detection reaction process, in accordance with the present invention, is best understood by referring to FIGS. **3**–**10**. It is described generally in WO 90/17239 to Barany et al., F. Barany et al., "Cloning, Overexpression and Nucleotide Sequence of a Thermostable DNA Ligase-encoding Gene," *Gene,* 109:1–11 (1991), and F. Barany, "Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase," *Proc. Natl. Acad. Sci. USA,* 88:189–193 (1991), the disclosures of which are hereby incorporated by reference. In accordance with the present invention, the ligase detection reaction can use 2 sets of complementary oligonucleotides. This is known as the ligase chain reaction which is described in the 3 immediately preceding references, which are hereby incorporated by reference. Alternatively, the ligase detection reaction can involve a single cycle which is known as the oligonucleotide ligation assay. See Landegren, et al., "A Ligase-Mediated Gene Detection Technique," *Science* 241:1077–80 (1988); Landegren, et al., "DNA Diagnostics—Molecular Techniques and Automation," Science 242:229–37 (1988); and U.S. Pat. No. 4,988,617 to Landegren, et al.

During the ligase detection reaction phase of the process, the denaturation treatment is carried out at a temperature of 80–105° C., while hybridization takes place at 50–85° C. Each cycle comprises a denaturation treatment and a thermal hybridization treatment which in total is from about one to five minutes long. Typically, the ligation detection reaction involves repeatedly denaturing and hybridizing for 2 to 50 cycles. The total time for the ligase detection reaction phase of the process is 1 to 250 minutes.

The oligonucleotide probe sets can be in the form of ribonucleotides, deoxynucleotides, modified ribonucleotides, modified deoxyribonucleotides, peptide nucleotide analogues, modified peptide nucleotide analogues, modified phosphate-sugar-backbone oligonucleotides, nucleotide analogs, and mixtures thereof.

In one variation, the oligonucleotides of the oligonucleotide probe sets each have a hybridization or melting temperature (i.e. T_m) of 66–70° C. These oligonucleotides are 20–28 nucleotides long.

It may be desirable to destroy chemically or enzymatically unconverted LDR oligonucleotide probes that contain addressable nucleotide array-specific portions prior to capture of the ligation products on a DNA array. Such unconverted probes will otherwise compete with ligation products for binding at the addresses on the array of the solid support which contain complementary sequences. Destruction can be accomplished by utilizing an exonuclease, such as exonuclease III (L-H Guo and R. Wu, *Methods in Enzymology* 100:60–96 (1985), which is hereby incorporated by reference) in combination with LDR probes that are blocked at the ends and not involved with ligation of probes to one another. The blocking moiety could be a reporter group or a phosphorothioate group. T. T. Nikiforow, et al., "The Use of Phosphorothioate Primers and Exonuclease Hydrolysis for the Preparation of Single-stranded PCR Products and their Detection by Solid-phase Hybridization," *PCR Methods and Applications,* 3:p. 285–291 (1994), which is hereby incorporated by reference. After the LDR process, unligated probes are selectively destroyed by incubation of the reaction mixture with the exonuclease. The ligated probes are protected due to the elimination of free 3' ends which are required for initiation of the exonuclease reaction. This approach results in an increase in the signal-to-noise ratio, especially where the LDR reaction forms only a small amount of product. Since unligated oligonucleotides compete for capture by the capture oligonucleotide, such competition with the ligated oligonucleotides lowers the signal. An additional advantage of this approach is that unhybridized label-containing sequences are degraded and, therefore, are less able to cause a target-independent background signal, because they can be removed more easily from the DNA array by washing.

The oligonucleotide probe sets, as noted above, have a reporter label suitable for detection. Useful labels include chromophores, fluorescent moieties, enzymes, antigens, heavy metals, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moieties, and electrochemical detecting moieties. The capture oligonucleotides can be in the form of ribonucleotides, deoxyribonucleotides, modified ribonucleotides, modified deoxyribonucleotides, peptide nucleotide analogues, modified peptide nucleotide analogues, modified phosphate-sugar backbone oligonucleotides, nucleotide analogues, and mixtures thereof. Where the process of the present invention involves use of a plurality of oligonucleotide sets, the second oligonucleotide probes can be the same, while the addressable array-specific portions of the first oligonucleotide probes differ. Alternatively, the addressable array-specific portions of the first oligonucleotide probes may be the same, while the reporter labels of the second oligonucleotide probes are different.

Prior to the ligation detection reaction phase of the present invention, the sample is preferably amplified by an initial target nucleic acid amplification procedure. This increases the quantity of the target nucleotide sequence in the sample. For example, the initial target nucleic acid amplification may

be accomplished using the polymerase chain reaction process, self-sustained sequence replication, or Q-β replicase-mediated RNA amplification. The polymerase chain reaction process is the preferred amplification procedure and is fully described in H. Erlich, et. al., "Recent Advances in the Polymerase Chain Reaction," *Science* 252: 1643–50 (1991); M. Innis, et. al., *PCR Protocols: A Guide to Methods and Applications*, Academic Press: New York (1990); and R. Saiki, et. al., "Primer-directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase," *Science* 239: 487–91 (1988), which are hereby incorporated by reference. J. Guatelli, et. al., "Isothermal, in vitro Amplification of Nucleic Acids by a Multienzyme Reaction Modeled After Retroviral Replication," *Proc. Natl. Acad. Sci. USA* 87: 1874–78 (1990), which is hereby incorporated by reference, describes the self-sustained sequence replication process. The Q-β replicase-mediated RNA amplification is disclosed in F. Kramer, et. al., "Replicatable RNA Reporters," *Nature* 339: 401–02 (1989), which is hereby incorporated by reference.

The use of the polymerase chain reaction process and then the ligase detection process, in accordance with the present invention, is shown in FIG. **3**. Here, homo- or heterozygosity at two polymorphisms (i.e. allele differences) are on the same gene. Such allele differences can alternatively be on different genes.

As shown in FIG. **3**, the target nucleic acid, when present in the form of a double stranded DNA molecule is denatured to separate the strands. This is achieved by heating to a temperature of 80–105° C. Polymerase chain reaction primers are then added and allowed to hybridize to the strands, typically at a temperature of 20–85° C. A thermostable polymerase (e.g., *Thermus aquaticus* polymerase) is also added, and the temperature is then adjusted to 50–85° C. to extend the primer along the length of the nucleic acid to which the primer is hybridized. After the extension phase of the polymerase chain reaction, the resulting double stranded molecule is heated to a temperature of 80–105° C. to denature the molecule and to separate the strands. These hybridization, extension, and denaturation steps may be repeated a number of times to amplify the target to an appropriate level.

Once the polymerase chain reaction phase of the process is completed, the ligation detection reaction phase begins, as shown in FIG. **3**. After denaturation of the target nucleic acid, if present as a double stranded DNA molecule, at a temperature of 80–105° C., preferably 94° C., ligation detection reaction oligonucleotide probes for one strand of the target nucleotide sequence are added along with a ligase (for example, as shown in FIG. **3**, a thermostable ligase like *Thermus aquaticus* ligase). The oligonucleotide probes are then allowed to hybridize to the target nucleic acid molecule and ligate together, typically, at a temperature of 45–85° C., preferably, 65° C. When there is perfect complementarity at the ligation junction, the oligonucleotides can be ligated together. Where the variable nucleotide is T or A, the presence of T in the target nucleotide sequence will cause the oligonucleotide probe with the addressable array-specific portion **Z1** to ligate to the oligonucleotide probe with the reporter label F, and the presence of A in the target nucleotide sequence will cause the oligonucleotide probe with the addressable array-specific portion **Z2** to ligate to the oligonucleotide probe with reporter label F. Similarly, where the variable nucleotide is A or G, the presence of T in the target nucleotide sequence will cause the oligonucleotide probe with addressable array-specific portion **Z4** to ligate to the oligonucleotide probe with the reporter label F, and the presence of C in the target nucleotide sequence will cause the oligonucleotide probe with the addressable array-specific portion **Z3** to ligate to the oligonucleotide probe with reporter label F. Following ligation, the material is again subjected to denaturation to separate the hybridized strands. The hybridization/ligation and denaturation steps can be carried through one or more cycles (e.g., 1 to 50 cycles) to amplify the target signal. Fluorescent ligation products (as well as unligated oligonucleotide probes having an addressable array-specific portion) are captured by hybridization to capture probes complementary to portions **Z1**, **Z2**, **Z3**, and **Z4** at particular addresses on the addressable arrays. The presence of ligated oligonucleotides is then detected by virtue of the label F originally on one of the oligonucleotides. In FIG. **3**, ligated product sequences hybridize to the array at addresses with capture oligonucleotides complementary to addressable array-specific portions **Z1** and **Z3**, while unligated oligonucleotide probes with addressable array-specific portions **Z2** and **Z4** hybridize to their complementary capture oligonucleotides. However, since only the ligated product sequences have label F, only their presence is detected.

FIG. **4** is a flow diagram of a PCR/LDR process, in accordance with the present invention, which distinguishes any possible base at a given site. Appearance of fluorescent signal at the addresses complementary to addressable array-specific portions **Z1**, **Z2**, **Z3**, and **Z4** indicates the presence of A, G, C, and T alleles in the target nucleotide sequence, respectively. Here, the presence of the A and C alleles in the target nucleotide sequences is indicated due to the fluorescence at the addresses on the solid support with capture oligonucleotide probes complementary to portions **Z1** and **Z3**, respectively. Note that in FIG. **4** the addressable array-specific portions are on the discriminating oligonucleotide probes, and the discriminating base is on the 3' end of these probes.

FIG. **5** is a flow diagram of a PCR/LDR process, in accordance with the present invention, for detecting the presence of any possible base at two nearby sites. Here, the LDR primers are able to overlap, yet are still capable of ligating provided there is perfect complementarity at the junction. This distinguishes LDR from other approaches, such as allele-specific PCR where overlapping primers would interfere with one another. In FIG. **5**, the first nucleotide position is heterozygous at the A and C alleles, while the second nucleotide position is heterozygous to the G, C, and T alleles. As in FIG. **4**, the addressable array-specific portions are on the discriminating oligonucleotide probes, and the discriminating base is on the 3' end of these probes. The reporter group (e.g., the fluorescent label) is on the 3' end of the common oligonucleotide probes. This is possible for example with the 21 hydroxylase gene, where each individual has 2 normal and 2 pseudogenes, and, at the intron 2 splice site (nucleotide 656), there are 3 possible single bases (G, A, and C). Also, this can be used to detect low abundance mutations in HIV infections which might indicate emergence of drug resistant (e.g., to AZT) strains. Returning to FIG. **5**, appearance of fluorescent signal at the addresses complementary to addressable array-specific portions **Z1**, **Z2**, **Z3**, **Z4**, **Z5**, **Z6**, **Z7**, and **Z8** indicates the presence of the A, G, C, and T, respectively, in the site heterozygous at the A and C alleles, and A, G, C, and T, respectively, in the site heterozygous at the G, C, and T alleles.

FIG. **6** is a flow diagram of a PCR/LDR process, in accordance with the present invention, where insertions (top left set of probes) and deletions (bottom right set of probes)

are distinguished. On the left, the normal sequence contains 5 A's in a polyA tract. The mutant sequence has an additional 2As inserted into the tract. Therefore, the LDR products with addressable array-specific portions Z1 (representing the normal sequence) and Z3 (representing a 2 base pair insertion) would be fluorescently labeled by ligation to the common primer. While the LDR process (e.g., using a thermostable ligase enzyme) has no difficulty distinguishing single base insertions or deletions in mononucleotide repeats, allele-specific PCR is unable to distinguish such differences, because the 3' base remains the same for both alleles. On the right, the normal sequence is a (CA)5 repeat (i.e. CACACACACA (SEQ. ID. No. 1)). The mutant contains two less CA bases than the normal sequence (i.e. CACACA). These would be detected as fluorescent LDR products at the addressable array-specific portions Z8 (representing the normal sequence) and Z6 (representing the 2 CA deletion) addresses. The resistance of various infectious agents to drugs can also be determined using the present invention. In FIG. **6**, the presence of ligated product sequences, as indicated by fluorescent label F, at the address having capture oligonucleotides complementary to Z1 and Z3 demonstrates the presence of both the normal and mutant poly A sequences. Similarly, the presence of ligated product sequences, as indicated by fluorescent label F, at the address having capture oligonucleotides complementary to Z6 and Z8 demonstrates the presence of both the normal CA repeat and a sequence with one repeat unit deleted.

FIG. **7** is a flow diagram of a PCR/LDR process, in accordance with the present invention, using addressable array-specific portions to detect a low abundance mutation in the presence of an excess of normal sequence. FIG. **7** shows codon 12 of the K-ras gene, sequence GGT, which codes for glycine ("Gly"). A small percentage of the cells contain the G to A mutation in GAT, which codes for aspartic acid ("Asp"). The LDR probes for wild-type (i.e. normal sequences) are missing from the reaction. If the normal LDR probes (with the discriminating base=G) were included, they would ligate to the common probes and overwhelm any signal coming from the mutant target. Instead, as shown in FIG. **7**, the existence of a ligated product sequence with fluorescent label F at the address with a capture oligonucleotide complementary to addressable array-specific portion Z4 indicates the presence of the aspartic acid encoding mutant.

FIG. **8** is a flow diagram of a PCR/LDR process, in accordance with the present invention, where the addressable array-specific portion is placed on the common oligonucleotide probe, while the discriminating oligonucleotide probe has the reporter label. Allele differences are distinguished by different fluorescent signals, F1, F2, F3, and F4. This mode allows for a more dense use of the arrays, because each position is predicted to light up with some group. It has the disadvantage of requiring fluorescent groups which have minimal overlap in their emission spectra and will require multiple scans. It is not ideally suitable for detection of low abundance alleles (e.g., cancer associated mutations).

FIG. **9** is a flow diagram of a PCR/LDR process, in accordance with the present invention, where both adjacent and nearby alleles are detected. The adjacent mutations are right next to each other, and one set of oligonucleotide probes discriminates the bases on the 3' end of the junction (by use of different addressable array-specific portions Z1, Z2, Z3, and Z4), while the other set of oligonucleotide probes discriminates the bases on the 5' end of the junction (by use of different fluorescent reporter labels F1, F2, F3, and F4). In FIG. **9**, codons in a disease gene (e.g. CFTR for cystic fibrosis) encoding Gly and arginine ("Arg"), respectively, are candidates for germline mutations. The detection results in FIG. **9** show the Gly (GGA; indicated by the ligated product sequence having portion Z2 and label F2) has been mutated to glutamic acid ("Glu") (GAA; indicated by the ligated product sequence having portion Z2 and label F1), and the Arg (CGG; indicated by the ligated product sequence having portion Z7 and label F2) has been mutated to tryptophan ("Trp") (TGG; indicated by the ligated product sequence with portion Z8 and label F2). Therefore, the patient is a compound heterozygous individual (i.e. with allele mutations in both genes) and will have the disease.

FIG. **10** is a flow diagram of a PCR/LDR process, in accordance with the present invention, where all possible single-base mutations for a single codon are detected. Most amino acid codons have a degeneracy in the third base, thus the first two positions can determine all the possible mutations at the protein level. These amino acids include arginine, leucine, serine, threonine, proline, alanine, glycine, and valine. However, some amino acids are determined by all three bases in the codon and, thus, require the oligonucleotide probes to distinguish mutations in 3 adjacent positions. By designing four oligonucleotide probes containing the four possible bases in the penultimate position to the 3' end, as well as designing an additional four capture oligonucleotides containing the four possible bases at the 3' end, as shown in FIG. **10**, this problem has been solved. The common oligonucleotide probes with the reporter labels only have two fluorescent groups which correspond to the codon degeneracies and distinguish between different ligated product sequences which are captured at the same array address. For example, as shown in FIG. **10**, the presence of a glutamine ("Gln") encoding codon (i.e., CAA and CAG) is indicated by the presence of a ligated product sequence containing portion Z1 and label F2. Likewise, the existence of a Gln to histidine ("His") encoding mutation (coded by the codon CAC) is indicated by the presence of ligated product sequences with portion Z1 and label F2 and with portion Z7 and label F2 There is an internal redundancy built into this assay due to the fact that primers Z1 and Z7 have the identical sequence.

A particularly important aspect of the present invention is its capability to quantify the amount of target nucleotide sequence in a sample. This can be achieved in a number of ways by establishing standards which can be internal (i.e. where the standard establishing material is amplified and detected with the sample) or external (i.e. where the standard establishing material is not amplified, and is detected with the sample).

In accordance with one quantification method, the signal generated by the reporter label, resulting from capture of ligated product sequences produced from the sample being analyzed, are detected. The strength of this signal is compared to a calibration curve produced from signals generated by capture of ligated product sequences in samples with known amounts of target nucleotide sequence. As a result, the amount of target nucleotide sequence in the sample being analyzed can be determined. This techniques involves use of an external standard.

Another quantification method, in accordance with the present invention, relates to an internal standard. Here, a known amount of one or more marker target nucleotide sequences are added to the sample. In addition, a plurality of marker-specific oligonucleotide probe sets are added along with the ligase, the previously-discussed oligonucleotide probe sets, and the sample to a mixture. The marker-specific oligonucleotide probe sets have (1) a first oligonucleotide

probe with a target-specific portion complementary to the marker target nucleotide sequence and an addressable array-specific portion complementary to capture oligonucleotides on the solid support and (2) a second oligonucleotide probe with a target-specific portion complementary to the marker target nucleotide sequence and a detectable reporter label. The oligonucleotide probes in a particular marker-specific oligonucleotide set are suitable for ligation together when hybridized adjacent to one another on a corresponding marker target nucleotide sequence. However, there is a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the sample or added marker sequences. The presence of ligated product sequences captured on the solid support is identified by detection of reporter labels. The amount of target nucleotide sequences in the sample is then determined by comparing the amount of captured ligated product generated from known amounts of marker target nucleotide sequences with the amount of other ligated product sequences captured.

Another quantification method in accordance with the present invention involves analysis of a sample containing two or more of a plurality of target nucleotide sequences with a plurality of sequence differences. Here, ligated product sequences corresponding to the target nucleotide sequences are detected and distinguished by any of the previously-discussed techniques. The relative amounts of the target nucleotide sequences in the sample are then quantified by comparing the relative amounts of captured ligated product sequences generated. This provides a quantitative measure of the relative level of the target nucleotide sequences in the sample.

The ligase detection reaction process phase of the present invention can be preceded by the ligase chain reaction process to achieve oligonucleotide product amplification. This process is fully described in F. Barany, et. al., "Cloning, Overexpression and Nucleotide Sequence of a Thermostable DNA Ligase-encoding Gene," *Gene* 109: 1–11 (1991) and F. Barany, "Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase," *Proc. Natl. Acad. Sci. USA* 88: 18993 (1991), which are hereby incorporated by reference. Instead of using the ligase chain reaction to achieve amplification, a transcription-based amplifying procedure can be used.

The preferred thermostable ligase is that derived from *Thermus aquaticus*. This enzyme can be isolated from that organism. M. Takahashi, et al., "Thermophillic DNA Ligase," *J. Biol. Chem.* 259:10041–47 (1984), which is hereby incorporated by reference. Alternatively, it can be prepared recombinantly. Procedures for such isolation as well as the recombinant production of *Thermus aquaticus* ligase as well as *Thermus themophilus* ligase) are disclosed in WO 90/17239 to Barany, et. al., and F. Barany, et al., "Cloning, Overexpression and Nucleotide Sequence of a Thermostable DNA-Ligase Encoding Gene," *Gene* 109: 1–11 (1991), which are hereby incorporated by reference. These references contain complete sequence information for this ligase as well as the encoding DNA. Other suitable ligases include *E. coli* ligase, T4 ligase, and *Pyrococcus* ligase.

The ligation amplification mixture may include a carrier DNA, such as salmon sperm DNA.

The hybridization step, which is preferably a thermal hybridization treatment, discriminates between nucleotide sequences based on a distinguishing nucleotide at the ligation junctions. The difference between the target nucleotide sequences can be, for example, a single nucleic acid base difference, a nucleic acid deletion, a nucleic acid insertion, or rearrangement. Such sequence differences involving more than one base can also be detected. Preferably, the oligonucleotide probe sets have substantially the same length so that they hybridize to target nucleotide sequences at substantially similar hybridization conditions. As a result, the process of the present invention is able to detect infectious diseases, genetic diseases, and cancer. It is also useful in environmental monitoring, forensics, and food science.

A wide variety of infectious diseases can be detected by the process of the present invention. Typically, these are caused by bacterial, viral, parasite, and fungal infectious agents. The resistance of various infectious agents to drugs can also be determined using the present invention.

Bacterial infectious agents which can be detected by the present invention include *Escherichia coli, Salmonella, Shigella, Klebsiella, Pseudomonas, Listeria monocytogenes, Mycobacterium tuberculosis, Mycobacterium avium-intracellulare, Yersinia, Francisella, Pasteurella, Brucella, Clostridia, Bordetella pertussis, Bacteroides, Staphylococcus aureus, Streptococcus pneumonia*, B-Hemolytic strep., *Corynebacteria, Legionella, Mycoplasma, Ureaplasma, Chlamydia, Neisseria gonorrhea, Neisseria meningitides, Hemophilus influenza, Enterococcus faecalis, Proteus vulgaris, Proteus mirabilis, Helicobacter pylori, Treponema palladium, Borrelia burgdorferi, Borrelia recurrentis, Rickettsial pathogens, Nocardia*, and *Acitnomycetes.*

Fungal infectious agents which can be detected by the present invention include *Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccicioides brasiliensis, Candida albicans, Aspergillus fumigautus, Phycomycetes* (*Rhizopus*), *Sporothrix schenckii, Chromomycosis*, and *Maduromycosis.*

Viral infectious agents which can be detected by the present invention include human immunodeficiency virus, human T-cell lymphocytotrophic virus, hepatitis viruses (e.g., Hepatitis B Virus and Hepatitis C Virus), Epstein-Barr Virus, cytomegalovirus, human papillomaviruses, orthomyxo viruses, paramyxo viruses, adenoviruses, corona viruses, rhabdo viruses, polio viruses, toga viruses, bunya viruses, arena viruses, rubella viruses, and reo viruses.

Parasitic agents which can be detected by the present invention include *Plasmodium falciparum, Plasmodium* malaria, *Plasmodium vivax, Plasmodium ovale, Onchoverva volvulus, Leishmania, Trypanosoma* spp., *Schistosoma* spp., *Entamoeba histolytica, Cryptosporidum, Giardia* spp., *Trichimonas* spp., *Balatidium coli, Wuchereria bancrofti, Toxoplasma* spp., *Enterobius vermicularis, Ascaris lumbricoides, Trichuris trichiura, Dracunculus medinesis*, trematodes, *Diphyllobothrium latum, Taenia* spp., *Pneumocystis carinii*, and *Necator americanis.*

The present invention is also useful for detection of drug resistance by infectious agents. For example, vancomycin-resistant *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae*, multi-drug resistant *Mycobacterium tuberculosis*, and AZT-resistant human immunodeficiency virus can all be identified with the present invention.

Genetic diseases can also be detected by the process of the present invention. This can be carried out by prenatal screening for chromosomal and genetic aberrations or post natal screening for genetic diseases. Examples of detectable genetic diseases include: 21 hydroxylase deficiency, cystic fibrosis, Fragile X Syndrome, Turner Syndrome, Duchenne Muscular Dystrophy, Down Syndrome or other trisomies, heart disease, single gene diseases, HLA typing, phenylketonuria, sickle cell anemia, Tay-Sachs Syndrome, thalassemia, Klinefelter's Syndrome, Huntington's Disease,

autoimmune diseases, lipidosis, obesity defects, hemophilia, inborn errors in metabolism, and diabetes.

Cancers which can be detected by the process of the present invention generally involve oncogenes, tumor suppressor genes, or genes involved in DNA amplification, replication, recombination, or repair. Examples of these include: BRCA1 gene, p53 gene, *Familial polyposis coli*, Her2/Neu amplification, Bcr/Ab1, K-ras gene, human papillomavirus Types 16 and 18, leukemia, colon cancer, breast cancer, lung cancer, prostate cancer, brain tumors, central nervous system tumors, bladder tumors, melanomas, liver cancer, osteosarcoma and other bone cancers, testicular and ovarian carcinomas, ENT tumors, and loss of heterozygosity.

In the area of environmental monitoring, the present invention can be used for detection, identification, and monitoring of pathogenic and indigenous microorganisms in natural and engineered ecosystems and microcosms such as in municipal waste water purification systems and water reservoirs or in polluted areas undergoing bioremediation. It is also possible to detect plasmids containing genes that can metabolize xenobiotics, to monitor specific target microorganisms in population dynamic studies, or either to detect, identify, or monitor genetically modified microorganisms in the environment and in industrial plants.

The present invention can also be used in a variety or forensic areas, including for human identification for military personnel and criminal investigation, paternity testing and family relation analysis, HLA compatibility typing, and screening blood, sperm, or transplantation organs for contamination.

In the food and feed industry, the present invention has a wide variety of applications. For example, it can be used for identification and characterization of production organisms such as yeast for production of beer, wine, cheese, yogurt, bread, etc. Another area of use is with regard to quality control and certification of products and processes (e.g., livestock, pasteurization, and meat processing) for contaminants. Other uses include the characterization of plants, bulbs, and seeds for breeding purposes, identification of the presence of plant-specific pathogens, and detection and identification of veterinary infections.

Desirably, the oligonucleotide probes are suitable for ligation together at a ligation junction when hybridized adjacent to one another on a corresponding target nucleotide sequence due to perfect complementarity at the ligation junction. However, when the oligonucleotide probes in the set are hybridized to any other nucleotide sequence present in the sample, there is a mismatch at a base at the ligation junction which interferes with ligation. Most preferably, the mismatch is at the base adjacent the 3' base at the ligation junction. Alternatively, the mismatch can be at the bases adjacent to bases at the ligation junction.

The process of the present invention is able to detect the first and second nucleotide sequences in the sample in an amount of 100 attomoles to 250 femtomoles. By coupling the LDR step with a primary polymerase-directed amplification step, the entire process of the present invention is able to detect target nucleotide sequences in a sample containing as few as a single molecule. Furthermore, PCR amplified products, which often are in the picomole amounts, may easily be diluted within the above range. The ligase detection reaction achieves a rate of formation of mismatched ligated product sequences which is less than 0.005 of the rate of formation of matched ligated product sequences.

Once the ligation phase of the process is completed, the capture phase is initiated. During the capture phase of the process, the mixture is contacted with the solid support at a temperature of 45–90° C. and for a time period of up to 60 minutes. Hybridizations may be accelerated by adding volume exclusion or chaotropic agents. When an array consists of dozens to hundreds of addresses, it is important that the correct ligation products have an opportunity to hybridize to the appropriate address. This may be achieved by the thermal motion of oligonucleotides at the high temperatures used, by mechanical movement of the fluid in contact with the array surface, or by moving the oligonucleotides across the array by electric fields. After hybridization, the array is washed sequentially with a low stringency wash buffer and then a high stringency wash buffer.

It is important to select capture oligonucleotides and addressable nucleotide sequences which will hybridize in a stable fashion. This requires that the oligonucleotide sets and the capture oligonucleotides be configured so that the oligonucleotide sets hybridize to the target nucleotide sequences at a temperature less than that which the capture oligonucleotides hybridize to the addressable array-specific portions. Unless the oligonucleotides are designed in this fashion, false positive signals may result due to capture of adjacent unreacted oligonucleotides from the same oligonucleotide set which are hybridized to the target.

The detection phase of the process involves scanning and identifying if ligation of particular oligonucleotide sets occurred and correlating ligation to a presence or absence of the target nucleotide sequence in the test sample. Scanning can be carried out by scanning electron microscopy, confocal microscopy, charge-coupled device, scanning tunneling electron microscopy, infrared microscopy, atomic force microscopy, electrical conductance, and fluorescent or phosphor imaging. Correlating is carried out with a computer.

Another aspect of the present invention relates to a method of forming an array of oligonucleotides on a solid support. This method involves providing a solid support having an array of positions each suitable for attachment of an oligonucleotide. A linker or support (preferably non-hydrolyzable), suitable for coupling an oligonucleotide to the solid support at each of the array positions, is attached to the solid support. An array of oligonucleotides on a solid support is formed by a series of cycles of activating selected array positions for attachment of multimer nucleotides and attaching multimer nucleotides at the activated array positions.

Yet another aspect of the present invention relates to an array of oligonucleotides on a solid support per se. The solid support has an array of positions each suitable for an attachment of an oligonucleotide. A linker or support (preferably non-hydrolyzable), suitable for coupling an oligonucleotide to the solid support, is attached to the solid support at each of the array positions. An array of oligonucleotides are placed on a solid support with at least some of the array positions being occupied by oligonucleotides having greater than sixteen nucleotides.

In the method of forming arrays, multimer oligonucleotides from different multimer oligonucleotide sets are attached at different array positions on a solid support. As a result, the solid support has an array of positions with different groups of multimer oligonucleotides attached at different positions.

The 1,000 different addresses can be unique capture oligonucleotide sequences (e.g., 24-mer) linked covalently to the target-specific sequence (e.g., approximately 20- to 25-mer) of a LDR oligonucleotide probe. A capture oligonucleotide probe sequence does not have any homology to either the target sequence or to other sequences on genomes

which may be present in the sample. This oligonucleotide probe is then captured by its addressable array-specific portion, a sequence complementary to the capture oligonucleotide on the addressable solid support array. The concept is shown in two possible formats, for example, for detection of the p53 R248 mutation (FIGS. **13**A–C).

In FIGS. **13**A–C, the top portion of the diagram shows two alternative formats for oligonucleotide probe design to identify the presence of a germ line mutation in codon 248 of the p53 tumor suppressor gene. The wild type sequence codes for arginine (R248), while the cancer mutation codes for tryptophan (R248W). The bottom part of the diagram is a schematic diagram of the capture oligonucleotide. The thick horizontal line depicts the membrane or solid surface containing the addressable array. The thin curved lines indicate a flexible linker arm. The thicker lines indicate a capture oligonucleotide sequence, attached to the solid surface in the C to N direction. For illustrative purposes, the capture oligonucleotides are drawn vertically, making the linker arm in section B appear "stretched". Since the arm is flexible, the capture oligonucleotide will be able to hybridize 5' to C and 3' to N in each case, as dictated by base pair complementarity. A similar orientation of oligonucleotide hybridization would be allowed if the oligonucleotides were attached to the membrane at the N-terminus. In this case, DNA/PNA hybridization would be in standard antiparallel 5' to 3' and 3' to 5'. Other modified sugar-phosphate backbones would be used in a similar fashion. FIGS. **13**A–B shows two LDR primers that are designed to discriminate wild type and mutant p53 by containing the discriminating base C or T at the 3' end. In the presence of the correct target DNA and Tth ligase, the discriminating probe is covalently attached to a common downstream oligonucleotide. The downstream oligonucleotide is fluorescently labeled. The discriminating oligonucleotides are distinguished by the presence of unique addressable array-specific portions, Z**1** and Z**2**, at each of their 5' ends. A black dot indicates that target dependent ligation has taken place. After ligation, oligonucleotide probes may be captured by their complementary addressable array-specific portions at unique addresses on the array. Both ligated and unreacted oligonucleotide probes are captured by the oligonucleotide array. Unreacted fluorescently labeled common primers and target DNA are then washed away at a high temperature (approximately 65° C. to 80° C.) and low salt. Mutant signal is distinguished by detection of fluorescent signal at the capture oligonucleotide complementary to addressable array-specific portion Z**1**, while wild type signal appears at the capture oligonucleotide complementary to addressable array-specific portion Z**2**. Heterozygosity is indicated by equal signals at the capture oligonucleotides complementary to addressable array-specific portions Z**1** and Z**2**. The signals may be quantified using a fluorescent imager. This format uses a unique address for each allele and may be preferred for achieving very accurate detection of low levels of signal (30 to 100 attomoles of LDR product). FIG. **13**C shows the discriminating signals may be quantified using a fluorescent imager. This format uses a unique address where oligonucleotide probes are distinguished by having different fluorescent groups, F**1** and F**2**, on their 5' end. Either oligonucleotide probe may be ligated to a common downstream oligonucleotide probe containing an addressable array-specific portion Z**1** on its 3' end. In this format, both wild type and mutant LDR products are captured at the same address on the array, and are distinguished by their different fluorescence. This format allows for a more efficient use of the array and may be preferred when trying to detect hundreds of potential germline mutations.

The solid support can be made from a wide variety of materials. The substrate may be biological, nonbiological, organic, inorganic, or a combination of any of these, existing as particles, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, slides, discs, membranes, etc. The substrate may have any convenient shape, such as a disc, square, circle, etc. The substrate is preferably flat but may take on a variety of alternative surface configurations. For example, the substrate may contain raised or depressed regions on which the synthesis takes place. The substrate and its surface preferably form a rigid support on which to carry out the reactions described herein. The substrate and its surface is also chosen to provide appropriate light-absorbing characteristics. For instance, the substrate may be a polymerized Langmuir Blodgett film, functionalized glass, Si, Ge, GaAs, GaP, SiO_2, SiN_4, modified silicon, or any one of a wide variety of gels or polymers such as (poly)tetrafluoroethylene, (poly)vinylidenedifluoride, polystyrene, polycarbonate, polyethylene, polypropylene, polyvinyl chloride, poly(methyl acrylate), poly(methyl methacrylate), or combinations thereof. Other substrate materials will be readily apparent to those of ordinary skill in the art upon review of this disclosure. In a preferred embodiment, the substrate is flat glass or single-crystal silicon.

According to some embodiments, the surface of the substrate is etched using well known techniques to provide for desired surface features. For example, by way of the formation of trenches, v-grooves, mesa structures, raised platforms, or the like, the synthesis regions may be more closely placed within the focus point of impinging light, be provided with reflective "mirror" structures for maximization of light collection from fluorescent sources, or the like.

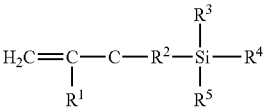
Surfaces on the solid substrate will usually, though not always, be composed of the same material as the substrate. Thus, the surface may be composed of any of a wide variety of materials, for example, polymers, plastics, ceramics, polysaccharides, silica or silica-based materials, carbon, metals, inorganic glasses, membranes, or composites thereof. The surface is functionalized with binding members which are attached firmly to the surface of the substrate. Preferably, the surface functionalities will be reactive groups such as silanol, olefin, amino, hydroxyl, aldehyde, keto, halo, acyl halide, or carboxyl groups. In some cases, such functionalities preexist on the substrate. For example, silica based materials have silanol groups, polysaccharides have hydroxyl groups, and synthetic polymers can contain a broad range of functional groups, depending on which monomers they are produced from. Alternatively, if the substrate does not contain the desired functional groups, such groups can be coupled onto the substrate in one or more steps.

A variety of commercially-available materials, which include suitably modified glass, plastic, or carbohydrate surfaces or a variety of membranes, can be used. Depending on the material, surface functional groups (e.g., silanol, hydroxyl, carboxyl, amino) may be present from the outset (perhaps as part of the coating polymer), or will require a separate procedure (e.g., plasma amination, chromic acid oxidation, treatment with a functionalized side chain alkyltrichlorosilane) for introduction of the functional group. Hydroxyl groups become incorporated into stable carbamate (urethane) linkages by several methods. Amino functions can be acylated directly, whereas carboxyl groups are activated, e.g., with N,N'-carbonyldiimidazole or water-soluble carbodiimides, and reacted with an amino-functionalized compound. As shown in FIG. **11**, the solid supports can be

membranes or surfaces with a starting functional group X. Functional group transformations can be carried out in a variety of ways (as needed) to provide group X* which represents one partner in the covalent linkage with group Y*. FIG. **11** shows specifically the grafting of PEG (i.e. polyethylene glycol), but the same repertoire of reactions can be used (however needed) to attach carbohydrates (with hydroxyl), linkers (with carboxyl), and/or oligonucleotides that have been extended by suitable functional groups (amino or carboxyl). In some cases, group X* or Y* is pre-activated (isolatable species from a separate reaction); alternatively, activation occurs in situ. Referring to PEG as drawn in FIG. **11**, Y and Y* can be the same (homobifunctional) or different (heterobifunctional); in the latter case, Y can be protected for further control of the chemistry. Unreacted amino groups will be blocked by acetylation or succinylation, to ensure a neutral or negatively charged environment that "repels" excess unhybridized DNA. Loading levels can be determined by standard analytical methods. Fields, et al., "Principles and Practice of Solid-Phase Peptide Synthesis," *Synthetic Peptides: A User's Guide*. G. Grant, Editor, W.H. Freeman and Co.: New York. p. 77–183 (1992), which is hereby incorporated by reference.

One approach to applying functional groups on a silica-based support surface is to silanize with a molecule either having the desired functional group (e.g., olefin, amino, hydroxyl, aldehyde, keto, halo, acyl halide, or carboxyl) or a molecule A able to be coupled to another molecule B containing the desired functional group. In the former case, functionalizing of glass- or silica-based solid supports with, for example, an amino group is carried out by reacting with an amine compound such as 3-aminopropyl triethoxysilane, 3-aminopropylmethyldiethoxysilane, 3-aminopropyl dimethylethoxysilane, 3-aminopropyl trimethoxysilane, N-(2-aminoethyl)-3-aminopropylmethyl dimethoxysilane, N-(2-aminoethyl-3-aminopropyl) trimethoxysilane, aminophenyl trimethoxysilane, 4-aminobutyldimethyl methoxysilane, 4-aminobutyl triethoxysilane, aminoethylaminomethyphenethyl trimethoxysilane, or mixtures thereof. In the latter case, molecule A preferably contains olefinic groups, such as vinyl, acrylate, methacrylate, or allyl, while molecule B contains olefinic groups and the desired functional groups. In this case, molecules A and B are polymerized together. In some cases, it is desirable to modify the silanized surface to modify its properties (e.g., to impart biocompatibility and to increase mechanical stability). This can be achieved by addition of olefinic molecule C along with molecule B to produce a polymer network containing molecules A, B, and C.

Molecule A is defined by the following formula:



R^1 is H or CH_3

R^2 is (C═O)—O—R^6, aliphatic group with or without functional substituent(s), an aromatic group with or without functional substituent(s), or mixed aliphatic/aromatic groups with or without functional substituent(s);

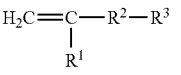
R^3 is an O-alkyl, alkyl, or halogen group;

R^4 is an O-alkyl, alkyl, or halogen group;

R^5 is an O-alkyl, alkyl, or halogen group; and

R^6 is an aliphatic group with or without functional substituent(s), an aromatic group with or without functional substituent(s), or mixed aliphatic/aromatic groups with or without functional substituent(s). Examples of Molecule A include 3(trimethoxysilyl) propyl methacrylate, N-[3-(trimethoxysilyl)propyl]-N'-(4-vinylbenzyl)ethylenediamine, triethoxyvinylsilane, triethylvinylsilane, vinyltrichlorosilane, vinyltrimethoxysilane, and vinylytrimethylsilane.

Molecule B can be any monomer containing one or more of the functional groups described above. Molecule B is defined by the following formula:



(i) R^1 is H or CH_3,

R^2 is (C═O), and

R^3 is OH or Cl.

or

(ii) R^1 is H or CH_3 and

R^2 is (C═O)—O—R^4, an aliphatic group with or without functional substituent(s), an aromatic group with or without functional substituent(s), and mixed aliphatic/aromatic groups with or without functional substituent(s); and

R^3 is a functional group, such as OH, COOH, NH_2, halogen, SH, COCI, or active ester; and

R^4 is an aliphatic group with or without functional substituent(s), an aromatic group with or without functional substituent(s), or mixed aliphatic/aromatic groups with or without functional substituent(s). Examples of molecule B include acrylic acid, acrylamide, methacrylic acid, vinylacetic acid, 4-vinylbenzoic acid, itaconic acid, allyl amine, allylethylamine, 4-aminostyrene, 2-aminoethyl methacrylate, acryloyl chloride, methacryloyl chloride, chlorostyrene, dichlorostyrene, 4-hydroxystyrene, hydroxymethyl styrene, vinylbenzyl alcohol, allyl alcohol, 2-hydroxyethyl methacrylate, or poly(ethylene glycol) methacrylate.

Molecule C can be any molecule capable of polymerizing to molecule A, molecule B, or both and may optionally contain one or more of the functional groups described above. Molecule C can be any monomer or cross-linker, such as acrylic acid, methacrylic acid, vinylacetic acid, 4-vinylbenzoic acid, itaconic acid, allyl amine, allylethylamine, 4-aminostyrene, 2-aminoethyl methacrylate, acryloyl chloride, methacryloyl chloride, chlorostyrene, dichlorostyrene, 4-hydroxystyrene, hydroxymethyl styrene, vinylbenzyl alcohol, allyl alcohol, 2-hydroxyethyl methacrylate, poly(ethylene glycol) methacrylate, methyl acrylate, methyl methacrylate, ethyl acrylate, ethyl methacrylate, styrene, 1-vinylimidazole, 2-vinylpyridine, 4-vinylpyridine, divinylbenzene, ethylene glycol dimethacryarylate, N,N'-methylenediacrylamide, N,N'-phenylenediacrylamide, 3,5-bis(acryloylamido)benzoic acid, pentaerythritol triacrylate, trimethylolpropane trimethacrylate, pentaerythritol tetraacrylate, trimethylolpropane ethoxylate (14/3 EO/OH) triacrylate, trimethyolpropane ethoxylate (7/3 EO/OH) triacrylate, triethylolpropane propoxylate (1 PO/OH) triacrylate, or trimethyolpropane propoxylate (2 PO/PH triacrylate).

Generally, the functional groups serve as starting points for oligonucleotides that will ultimately be coupled to the support. These functional groups can be reactive with an organic group that is to be attached to the solid support or it can be modified to be reactive with that group, as through the use of linkers or handles. The functional groups can also impart various desired properties to the support.

After functionalization (if necessary) of the solid support, tailor-made polymer networks containing activated functional groups that may serve as carrier sites for complementary oligonucleotide capture probes can be grafted to the support. The advantage of this approach is that the loading capacity of capture probes can thus be increased significantly, while physical properties of the intermediate solid-to-liquid phase can be controlled better. Parameters that are subject to optimization include the type and concentration of functional group-containing monomers, as well as the type and relative concentration of the crosslinkers that are used.

The surface of the functionalized substrate is preferably provided with a layer of linker molecules, although it will be understood that the linker molecules are not required elements of the invention. The linker molecules are preferably of sufficient length to permit polymers in a completed substrate to interact freely with molecules exposed to the substrate. The linker molecules should be 6–50 atoms long to provide sufficient exposure. The linker molecules may be, for example, aryl acetylene, ethylene glycol oligomers containing 2–10 monomer units, diamines, diacids, amino acids, or combinations thereof.

According to alternative embodiments, the linker molecules are selected based upon their hydrophilic/hydrophobic properties to improve presentation of synthesized polymers to certain receptors. For example, in the case of a hydrophilic receptor, hydrophilic linker molecules will be preferred to permit the receptor to approach more closely the synthesized polymer.

According to another alternative embodiment, linker molecules are also provided with a photocleavable group at any intermediate position. The photocleavable group is preferably cleavable at a wavelength different from the protective group. This enables removal of the various polymers following completion of the syntheses by way of exposure to the different wavelengths of light.

The linker molecules can be attached to the substrate via carbon—carbon bonds using, for example, (poly)tri-fluorochloroethylene surfaces or, preferably, by siloxane bonds (using, for example, glass or silicon oxide surfaces). Siloxane bonds with the surface of the substrate may be formed in one embodiment via reactions of linker molecules bearing trichlorosilyl groups. The linker molecules may optionally be attached in an ordered array, i.e., as parts of the head groups in a polymerized monolayer. In alternative embodiments, the linker molecules are adsorbed to the surface of the substrate.

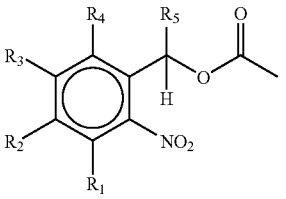
It is often desirable to introduce a PEG spacer with complementary functionalization, prior to attachment of the starting linker for DNA or PNA synthesis. G. Barany, et al., "Novel Polyethylene Glycol-polystyrene (PEG-PS) Graft Supports for Solid-phase Peptide Synthesis," ed. C. H. Schneider and A. N. Eberle., Leiden, The Netherlands: Escom Science Publishers. 267–268 (1993); Zalipsky, et al., "Preparation and Applications of Polyethylene Glycol-polystyrene Graft Resin Supports for Solid-phase Peptide Synthesis," *Reactive Polymers,* 22:243–58 (1994); J. M. Harris, ed. "Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications," (1992), Plenum Press: New York, which are hereby incorporated by reference. Similarly, dextran layers can be introduced as needed. Cass, et al., "Pilot, A New Peptide Lead Optimization Technique and Its Application as a General Library Method, in Peptides—Chemistry, Structure and Biology: Proceedings of the Thirteenth American Peptide Symposium", R. S. Hodges and J. A. Smith, Editor. (1994), Escom: Leiden, The Netherlands; Lofas, et al., "A Novel Hydrogel Matrix on Gold Surface Plasma Resonance Sensors for Fast and Efficient Covalent Immobilization of Ligands," *J. Chem. Soc., Chem. Commun.*, pp. 1526–1528 (1990), which are hereby incorporated by reference. Particularly preferred linkers are tris(alkoxy) benzyl carbonium ions with dilute acid due to their efficient and specific trapping with indole moieties. DNA oligonucleotides can be synthesized and terminated with a residue of the amino acid tryptophan, and conjugated efficiently to supports that have been modified by tris(alkoxy)benzyl ester (hypersensitive acid labile ("HAL")) or tris(alkoxy)benzylamide ("PAL") linkers [F. Albericio, et al., *J. Org. Chem.,* 55:3730–3743 (1990); F. Albericio and G. Barany, *Tetrahedron Lett.,* 32:1015–1018 (1991)], which are hereby incorporated by reference). Other potentially rapid chemistries involve reaction of thiols with bromoacetyl or maleimido functions. In one variation, the terminus of amino functionalized DNA is modified by bromoacetic anhydride, and the bromoacetyl function is captured by readily established thiol groups on the support. Alternatively, an N-acetyl, S-tritylcysteine residue coupled to the end of the probe provides, after cleavage and deprotection, a free thiol which can be captured by a maleimido group on the support. As shown in FIG. **12**, chemically synthesized probes can be extended, on either end. Further variations of the proposed chemistries are readily envisaged. FIG. **12**A shows that an amino group on the probe is modified by bromoacetic anhydride; the bromoacetyl function is captured by a thiol group on the support. FIG. **12**B shows that an N-acetyl, S-tritylcysteine residue coupled to the end of the probe provides, after cleavage and deprotection, a free thiol which is captured by a maleimido group on the support. FIG. **12**C shows a probe containing an oligotryptophanyl tail (n=1 to 3), which is captured after treatment of a HAL-modified solid support with dilute acid.

To prepare the arrays of the present invention, the solid supports must be charged with DNA oligonucleotides or PNA oligomers. This is achieved either by attachment of pre-synthesized probes, or by direct assembly and side-chain deprotection (without release of the oligomer) onto the support. Further, the support environment needs to be such as to allow efficient hybridization. Toward this end, two factors may be identified: (i) sufficient hydrophilic character of support material (e.g., PEG or carbohydrate moieties) and (ii) flexible linker arms (e.g., hexaethylene oxide or longer PEG chains) separating the probe from the support backbone. It should be kept in mind that numerous ostensibly "flat surfaces" are quite thick at the molecular level. Lastly, it is important that the support material not provide significant background signal due to non-specific binding or intrinsic fluorescence.

The linker molecules and monomers used herein are provided with a functional group to which is bound a protective group. Preferably, the protective group is on the distal or terminal end of the linker molecule opposite the substrate. The protective group may be either a negative protective group (i.e., the protective group renders the linker molecules less reactive with a monomer upon exposure) or a positive protective group (i.e., the protective group renders the linker molecules more reactive with a monomer upon exposure). In the case of negative protective groups, an

additional step of reactivation will be required. In some embodiments, this will be done by heating.

The protective group on the linker molecules may be selected from a wide variety of positive light-reactive groups preferably including nitro aromatic compounds such as o-nitrobenzyl derivatives or benzylsulfonyl. In a preferred embodiment, 6-nitroveratryloxycarbonyl ("NVOC"), 2-nitrobenzyloxycarbonyl ("NBOC"), Benzyloxycarbonyl ("BOC"), fluorenylmethoxycarbonyl ("FMOC"), or α,α-dimethyl-dimethoxybenzyloxycarbonyl ("DDZ") is used. In one embodiment, a nitro aromatic compound containing a benzylic hydrogen ortho to the nitro group is used, i.e., a chemical of the form:



where R_1 is alkoxy, alkyl, halo, aryl, alkenyl, or hydrogen; R_2 is alkoxy, alkyl, halo, aryl, nitro, or hydrogen; R_3 is alkoxy, alkyl, halo, nitro, aryl, or hydrogen; R_4 is alkoxy, alkyl, hydrogen, aryl, halo, or nitro; and R_5 is alkyl, alkynyl, cyano, alkoxy, hydrogen, halo, aryl, or alkenyl. Other materials which may be used include o-hydroxy-α-methyl cinnamoyl derivatives. Photoremovable protective groups are described in, for example, Patchornik, *J. Am. Chem. Soc.* 92:6333 (1970) and Amit et al., *J. Org. Chem.* 39:192 (1974), both of which are hereby incorporated by reference.

In an alternative embodiment, the positive reactive group is activated for reaction with reagents in solution. For example, a 5-bromo-7-nitro indoline group, when bound to a carbonyl, undergoes reaction upon exposure to light at 420 nm.

In a second alternative embodiment, the reactive group on the linker molecule is selected from a wide variety of negative light-reactive groups including a cinnamete group.

Alternatively, the reactive group is activated or deactivated by electron beam lithography, x-ray lithography, or any other radiation. A suitable reactive group for electron beam lithography is a sulfonyl group. Other methods may be used including, for example, exposure to a current source. Other reactive groups and methods of activation may be used in view of this disclosure.

The linking molecules are preferably exposed to, for example, light through a suitable mask using photolithographic techniques of the type known in the semiconductor industry and described in, for example, Sze, *VLSI Technology*, McGraw-Hill (1983), and Mead et al., *Introduction to VLSI Systems*, Addison-Wesley (1980), which are hereby incorporated by reference for all purposes. The light may be directed at either the surface containing the protective groups or at the back of the substrate, so long as the substrate is transparent to the wavelength of light needed for removal of the protective groups.

The mask is in one embodiment a transparent support material selectively coated with a layer of opaque material. Portions of the opaque material are removed, leaving opaque material in the precise pattern desired on the substrate surface. The mask is brought directly into contact with the substrate surface. "Openings" in the mask correspond to locations on the substrate where it is desired to remove photoremovable protective groups from the substrate. Alignment may be performed using conventional alignment techniques in which alignment marks are used accurately to overlay successive masks with previous patterning steps, or more sophisticated techniques may be used. For example, interferometric techniques such as the one described in Flanders et al., "A New Interferometric Alignment Technique." *App. Phys. Lett.* 31:426–428 (1977), which is hereby incorporated by reference, may be used.

To enhance contrast of light applied to the substrate, it is desirable to provide contrast enhancement materials between the mask and the substrate according to some embodiments. This contrast enhancement layer may comprise a molecule which is decomposed by light such as quinone diazide or a material which is transiently bleached at the wavelength of interest. Transient bleaching of materials will allow greater penetration where light is applied, thereby enhancing contrast. Alternatively, contrast enhancement may be provided by way of a cladded fiber optic bundle.

The light may be from a conventional incandescent source, a laser, a laser diode, or the like. If non-collimated sources of light are used, it may be desirable to provide a thick- or multi-layered mask to prevent spreading of the light onto the substrate. It may, further, be desirable in some embodiments to utilize groups which are sensitive to different wavelengths to control synthesis. For example, by using groups which are sensitive to different wavelengths, it is possible to select branch positions in the synthesis of a polymer or eliminate certain masking steps.

Alternatively, the substrate may be translated under a modulated laser or diode light source. Such techniques are discussed in, for example, U.S. Pat. No. 4,719,615 to Feyrer et al., which is hereby incorporated by reference. In alternative embodiments, a laser galvanometric scanner is utilized. In other embodiments, the synthesis may take place on or in contact with a conventional liquid crystal (referred to herein as a "light valve") or fiber optic light sources. By appropriately modulating liquid crystals, light may be selectively controlled to permit light to contact selected regions of the substrate. Alternatively, synthesis may take place on the end of a series of optical fibers to which light is selectively applied. Other means of controlling the location of light exposure will be apparent to those of skill in the art.

The development of linkers and handles for peptide synthesis is described in Fields, et al., "Principles and Practice of Solid-Phase Peptide Synthesis, "*Synthetic Peptides: A User's Guide*, G. Grant, Editor. W.H. Freeman and Co.: New York. p. 77–183 (1992); G. Barany, et al., "Recent Progress on Handles and Supports for Solid-phase Peptide Synthesis", *Peptides-Chemistry, Structure and Biology: Proceedings of the Thirteenth American Peptide Symposium*. R. S. Hodges and J. A. Smith, Editor. Escom Science Publishers: Leiden, The Netherlands pp. 1078–80 (1994), which are hereby incorporated by reference. This technology is readily extendable to DNA and PNA. Of particular interest is the development of PAL (Albericio, et al., "Preparation and Application of the 5-(4-(9-Fluorenylmethyloxycarbonyl)Aminomethyl-3,5-Dimethoxyphenoxy)Valeric Acid (PAL) Handle for the Solid-phase Synthesis of C-terminal Peptide Amides under Mild Conditions," *J. Org. Chem.,* 55:3730–3743 (1990), which is hereby incorporated by reference, and ester (HAL) (Albericio, et al., "Hypersensitive Acid-labile (HAL) Tris(alkoxy)Benzyl Ester Anchoring for Solid-phase Synthesis of Protected Peptide Segments," *Tetrahedron Lett.,* 32:1015–1018 (1991), which is hereby incorporated by reference, linkages, which upon cleavage with acid provide, respectively, C-terminal peptide amides, and protected peptide acids that can be used as building blocks for so-called segment condensation approaches. The stabilized carbonium ion generated in acid from cleavage of PAL or HAL linkages can be intercepted by

tryptophanyl-peptides. While this reaction is a nuisance for peptide synthesis and preventable (in part) by use of appropriate scavengers, it has the positive application of chemically capturing oligo-Trp-end-labelled DNA and PNA molecules by HAL-modified surfaces.

The art recognizes several approaches to making oligonucleotide arrays. Southern, et al., "Analyzing and Comparing Nucleic Acid Sequences by Hybridization to Arrays of Oligonucleotides: Evaluation using Experimental Models," *Genomics,* 13:1008–1017 (1992); Fodor, et al., "Multiplexed Biochemical Assays with Biological Chips," *Nature,* 364:555–556 (1993); Khrapko, et al., "A Method for DNA Sequencing by Hybridization with Oligonucleotide Matrix," *J. DNA Seq. Map.,* 1:375–388 (1991); Van Ness, et al., "A Versatile Solid Support System for Oligodeoxynucleoside Probe-based Hybridization Assays," *Nucleic Acids Res.,* 19:3345–3350 (1991); Zhang, et al., "Single-base Mutational Analysis of Cancer and Genetic Diseases Using Membrane Bound Modified Oligonucleotides," *Nucleic Acids Res.,* 19:3929–3933 (1991); K. Beattie, "Advances in Genosensor Research," *Clin. Chem.* 41(5):700–06 (1995), which are hereby incorporated by reference. These approaches may be divided into three categories: (i) Synthesis of oligonucleotides by standard methods and their attachment one at a time in a spatial array; (ii) Photolithographic masking and photochemical deprotection on a silicon chip, to allow for synthesis of short oligonucleotides (Fodor, et al., "Multiplexed Biochemical Assays with Biological Chips," *Nature,* 364:555–556 (1993) and R. J. Lipshutz, et al., "Using Oligonucleotide Probe Arrays To Assess Genetic Diversity," *Biotechniques* 19:442–447 (1995), which are hereby incorporated by reference); and (iii) Physical masking to allow for synthesis of short oligonucleotides by addition of single bases at the unmasked areas (Southern, et al., "Analyzing and Comparing Nucleic Acid Sequences by Hybridization to Arrays of Oligonucleotides: Evaluation Using Experimental Models," *Genomics,* 13:10081017 (1992); Maskos, et al., "A Study of Oligonucleotide Reassociation Using Large Arrays of Oligonucleotides Synthesised on a Glass Support," *Nucleic Acids Res.,* 21:4663–4669 (1993), which are hereby incorporated by reference).

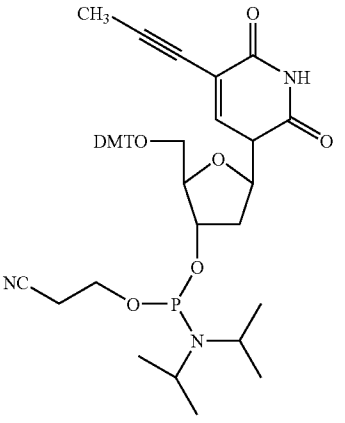
Although considerable progress has been made in constructing oligonucleotide arrays, some containing as many as 256 independent addresses, these procedures are less preferred, for detecting specific DNA sequences by hybridizations. More particularly, arrays containing longer oligonucleotides can currently be synthesized only by attaching one address at a time and, thus, are limited in potential size. Current methods for serially attaching an oligonucleotide take about 1 hour, thus an array of 1,000 addresses would require over 40 days of around-the-clock work to prepare. Arrays containing short oligonucleotides of 8- to 10-mers do not have commercial applicability, because longer molecules are needed to detect single base differences effectively.

These prior procedures may still be useful to prepare said supports carrying an array of oligonucleotides for the method of detection of the present invention. However, there are more preferred approaches.

It is desirable to produce a solid support with a good loading of oligonucleotide or PNA oligomer in a relatively small, but well-defined area. Current, commercially available fluorescent imagers can detect a signal as low as 3 attomoles per 50 μm square pixel. Thus, a reasonable size address or "spot" on an array would be about 4×4 pixels, or 200 μm square. Smaller addresses could be used with CCD detection. The limit of detection for such an address would be about 48 attomoles per "spot", which is comparable to the 100 attomole detection limit using a fluorescent DNA sequencing machine. The capacity of oligonucleotides which can be loaded per 200 μm square will give an indication of the potential signal to noise ratio. A loading of 20 fmoles would give a signal to noise ratio of about 400 to 1, while 200 fmoles would allow for a superb signal to noise ratio of about 4000 to 1. The oligonucleotide or PNA oligomer should be on a flexible "linker arm" and on the "outside" or "surface" of the solid support for easier hybridizations. The support should be non-fluorescent, and should not interfere with hybridization nor give a high background signal due to nonspecific binding.

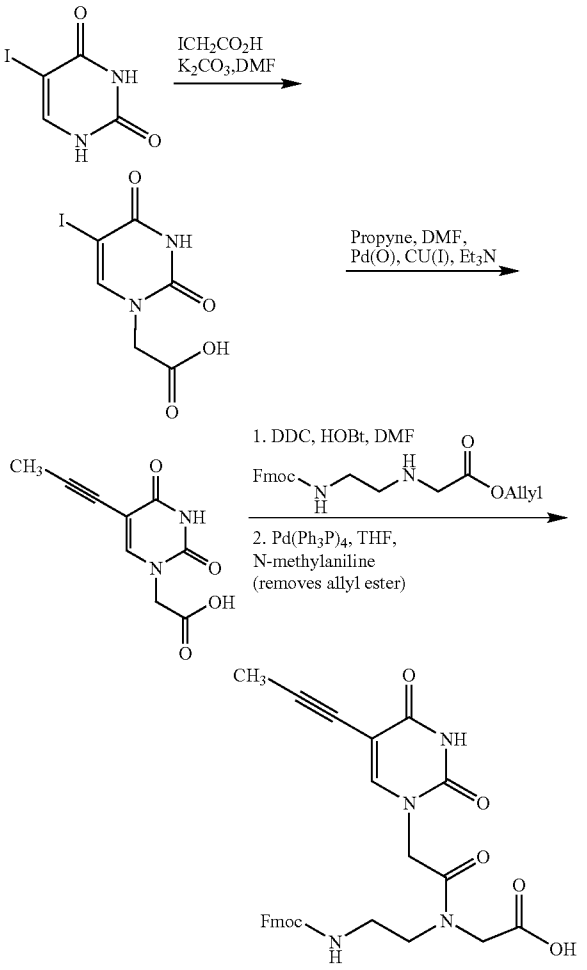
The complementary capture oligonucleotide addresses on the solid supports can be either DNA or PNA. PNA-based capture is preferred over DNA-based capture, because PNA/DNA duplexes are much stronger than DNA/DNA duplexes, by about 1° C./base-pair. M. Egholm, et al., "PNA Hybridizes to Complementary Oligonucleotides Obeying the Watson-Crick Hydrogen-bonding Rules," *Nature,* 365:566–568 (1993), which is hereby incorporated by reference. Thus, for a 24-mer DNA/DNA duplex with T_m=72° C., the corresponding duplex with one PNA strand would have a "predicted" T_m=96° C. (the actual melting point might be slightly lower as the above "rule of thumb" is less accurate as melting points get over 80° C.). Additionally, the melting difference between DNA/DNA and PNA/DNA becomes even more striking at low salt.

The melting temperature of DNA/DNA duplexes can be estimated as [4n(G·C)+2m(A·T)]° C. Oligonucleotide capture can be optimized by narrowing the T_m difference between duplexes formed by capture oligonucleotides and the complementary addressable array-specific portions hybridized to one another resulting from differences in G·C/A·T content. Using 5-propynyl-dU in place of thymine increases the T_m of DNA duplexes an average of 1.7° C. per substitution. Froehler, et al., "Oligonucleotides Containing C-5 Propyne Analogs of 2'-deoxyuridine and 2'-deoxycytidine," *Tetrahedron Lett.,* 33:5307–5310 (1992) and J. Sagi, et al., *Tetrahedron Letters,* 34:2191 (1993), which are hereby incorporated by reference. The same substitution in the capture scheme should lower the T_m difference between the components of such duplexes and raise the T_m for all of the duplexes. Phosphoramidite derivatives of 5-propynyl-dU having the following structure can be prepared according to the immediately preceding Froehler and Sagi references, which are hereby incorporated by reference.



The 5-propynyluracil PNA monomer with Fmoc amino protection can be made by the following synthesis (where

DMF is N,N'-dimethylformamide, DCC is N,N'-dicyclohexylcarbodiimide, HOBt is 1-hydroxybenzotriazole, and THF is tetrahydrofuran):



Using the methods described by Egholm, et al., "Peptide Nucleic Acids (PNA). Oligonucleotide Analogues with an Achiral Peptide Backbone," *J. Am. Chem. Soc.,* 114:1895–1897 (1992) and Egholm, et al., "Recognition of Guanine and Adenine in DNA by Cytosine and Thymine Containing Peptide Nucleic Acids (PNA)," *J. Am. Chem. Soc.,* 114:9677–9678 (1992), which are hereby incorporated by reference. The synthesis scheme above describes the preparation of a PNA monomer having a 5-propynyl-uracil base component. 5-Iodouracil is first alkylated with iodoacetic acid, and, then, the propynl group is coupled to the base moiety by a Pd/Cu catalyst. The remaining steps in the scheme follow from the above-referenced methods. These monomers can be incorporated into synthetic DNA and PNA strands.

There are two preferred general approaches for synthesizing arrays. In the first approach, full-length DNA oligonucleotides or PNA oligomers are prepared and are subsequently linked covalently to a solid support or membrane. In the second approach, specially designed PNA oligomers or DNA oligonucleotides are constructed by sequentially adding multimers to the solid support. These multimers are added to specific rows or columns on a solid support or membrane surface. The resulting "checkerboard" pattern generates unique addressable arrays of full length PNA or DNA.

FIGS. **14**–**16** show different modes of preparing full-length DNA oligonucleotides or PNA oligomers and, subsequently, linking those full length molecules to the solid support.

FIGS. **14**A–E depict a method for constructing an array of DNA or PNA oligonucleotides by coupling individual full-length oligonucleotides to the appropriate locations in a grid. The array of FIG. **14**A shows the pattern of oligonucleotides that would be generated if oligonucleotides are coupled to sixteen 200 μm×200 μm regions of the array surface. Each individual 200 μm×200 μm region contains DNA or PNA with a unique sequence which is coupled to the surface. The individual square regions will be separated from adjacent squares by 200 μm. The array of FIG. **14**A can thus support 64 (8×8) different oligonucleotides in an 3 mm by 3 mm area. In order to multiplex the construction of the array, 16 squares separated by a distance of 800 μm will be coupled simultaneously to their specific oligonucleotides. Therefore, the 8×8 grid could be constructed with only 4 machine steps as shown in FIGS. **14**B–**14**E. In these diagrams, the dark squares represent locations that are being altered in the current synthesis step, while the hatched squares represent regions which have been synthesized in earlier steps. The first step (FIG. **14**B) would immobilize oligonucleotides at locations A**1**, E**1**, I**1**, M**1**, A**5**, E**5**, **15**, M**5**, A**9**, E**9**, **19**, M**9**, A**13**, E**13**, **113**, and M**13** simultaneously. In the next step (FIG. **14**C), the machine would be realigned to start in Column C. After having completed Row **1**, the next step (FIG. **14**D) would start at Row 3. Finally, the last 16 oligonucleotides would be immobilized in order to complete the 8×8 grid (FIG. **14**E). Thus, the construction of the 8×8 array could be reduced to 4 synthesis steps instead of 64 individual spotting reactions. This method would be easily extended and an apparatus capable of spotting 96 oligomers simultaneously could be used rapidly to construct larger arrays.

FIGS. **15**A–E represent a perspective dimensional view of the array construction process described in FIG. **14**. In FIGS. **15**A–E, the construction of a 4×4 (16) array using a machine capable of spotting four different 24-mers simultaneously is depicted. First, as shown in FIG. **15**A, the machine attaches 4 oligomers at locations A**1**, E**1**, A**5**, and E**5**. Next, as shown in FIG. **15**B, the machine is shifted horizontally and attaches 4 oligomers at locations C**1**, G**1**, C**5**, and G**5**. Next, as shown in FIG. **15**C, the machine is repositioned and attaches 4 oligomers at locations A**3**, E**3**, A**7**, and E**7**. Finally, as shown in FIG. **15**D, the machine attaches the 4 remaining oligomers at positions C**3**, G**3**, C**7**, and G**7**. The completed array contains sixteen 24-mers as shown in the perspective view of FIG. **15**E.

FIGS. **16**A–C show views for an application apparatus 2 capable of simultaneously coupling 16 different oligonucleotides to different positions on an array grid G as shown in FIG. **14**. The black squares in the top view (FIG. **16**A) represent sixteen 200 μm×200 μm regions that are spatially separated from each other by 600 μm. The apparatus shown has 16 input ports which would allow tubes containing different oligonucleotides to fill the funnel-shaped chambers above different locations on the array. The side views (FIGS. **16**B–C, taken along lines **16**B—**16**B and **16**C—**16**C, of FIG. **16**A, respectively) of the apparatus demonstrate that funnel shaped chambers **4** align with the appropriate region on the array below the apparatus. In addition, two valves **6** and **8** (hatched squares in FIGS. **16**A–C) control the flow of

fluid in the 16 independent reaction chambers. One valve **6** controls the entry of fluids from the input port **10**, while the other valve **8** would be attached to a vacuum line **12** in order to allow loading and clearing of the reaction chamber **4**. The apparatus would first be aligned over the appropriate 200 μm×200 μm regions of the array. Next, the entire apparatus is firmly pressed against the array, thus forming a closed reaction chamber above each location. The presence of raised 10 μm ridges R around each location on the array ensures the formation of a tight seal which would prevent leakage of oligomers to adjacent regions on the array. Next, the valve **8** to vacuum line **12** would be opened, while the valve **10** from the input solution port **10** would be closed. This would remove air from the reaction chamber **4** and would create a negative pressure in the chamber. Then, the valve **8** to vacuum line **12** would be closed and the valve **10** from the input solution port **10** would be opened. The solution would flow into the reaction chamber **4** due to negative pressure. This process eliminates the possibility of an air bubble forming within the reaction chamber **4** and ensures even distribution of oligonucleotides across the 200 μm×200 μm region. After the oligonucleotides have been coupled to the activated array surface, the input valve **6** would be closed, the valve **8** to vacuum line **12** would be opened, and the apparatus would be lifted from the array surface in order to remove completely any excess solution from the reaction chamber. A second apparatus can now be realigned for the synthesis of the next 16 locations on the array.

FIGS. **15** to **26** show different modes of constructing PNA oligomers or DNA oligonucleotides on a solid support by sequentially adding, respectively, PNA or DNA, multimers to the solid support.

As an example of assembling arrays with multimers, such assembly can be achieved with tetramers. Of the 256 (4^4) possible ways in which four bases can be arranged as tetramers, 36 that have unique sequences can be selected. Each of the chosen tetramers differs from all the others by at least two bases, and no two dimers are complementary to each other. Furthermore, tetramers that would result in self-pairing or hairpin formation of the addresses have been eliminated.

The final tetramers are listed in Table 1 and have been numbered arbitrarily from 1 to 36. This unique set of tetramers are used as design modules for the sometimes desired 24-mer capture oligonucleotide address sequences. The structures can be assembled by stepwise (one base at a time) or convergent (tetramer building blocks) synthetic strategies. Many other sets of tetramers may be designed which follow the above rules. The segment approach is not uniquely limited to tetramers, and other units, i.e. dimers, trimers, pentamers, or hexamers could also be used.

TABLE 1

List of tetramer PNA sequences and complementary DNA sequences, which differ from each other by at least 2 bases.

Number	Sequence (N-C)	Complement (5'-3')	G + C
1.	TCTG	CAGA	2
2.	TGTC	GACA	2
3.	TCCC	GGGA	3
4.	TGCG	CGCA	3
5.	TCGT	ACGA	2
6.	TTGA	TCAA	1
7.	TGAT	ATCA	1
8.	TTAG	CTAA	1
9.	CTTG	CAAG	2
10.	CGTT	AACG	2
11.	CTCA	TGAG	2
12.	CACG	CGTG	3
13.	CTGT	ACAG	2
14.	CAGC	GCTG	3
15.	CCAT	ATGG	2
16.	CGAA	TTCG	2
17.	GCTT	AAGC	2
18.	GGTA	TACC	2
19.	GTCT	AGAC	2
20.	GACC	GGTC	3
21.	GAGT	ACTC	2
22.	GTGC	GCAC	3
23.	GCAA	TTGC	2
24.	GGAC	GTCC	3
25.	AGTG	CACT	2
26.	AATC	GATT	1
27.	ACCT	AGGT	2
28.	ATCG	CGAT	2
29.	ACGG	CCGT	3
30.	AGGA	TCCT	2
31.	ATAC	GTAT	1
32.	AAAG	CTTT	1
33.	CCTA	TAGG	2
34.	GATG	CATC	2
35.	AGCC	GGCT	3
36.	TACA	TGTA	1

Note that the numbering scheme for tetramers permits abbreviation of each address as a string of six numbers (e.g., second column of Table 2 infra). The concept of a 24-mer address designed from a unique set of 36 tetramers (Table 1) allows a huge number of possible structures, 36^6=2,176,782,336.

FIG. **17** shows one of the many possible designs of 36 tetramers which differ from each other by at least 2 bases. The checkerboard pattern shows all 256 possible tetramers. A given square represents the first two bases on the left followed by the two bases on the top of the checkerboard. Each tetramer must differ from each other by at least two bases, and should be non-complementary. The tetramers are shown in the white boxes, while their complements are listed as (number). Thus, the complementary sequences GACC (20) and GGTC (20') are mutually exclusive in this scheme. In addition, tetramers must be non-palindromic, e.g., TCGA (darker diagonal line boxes), and non-repetitive, e.g., CACA (darker diagonal line boxes from upper left to lower right). All other sequences which differ from the 36 tetramers by only 1 base are shaded in light gray. Four potential tetramers (white box) were not chosen as they are either all A·T or G·C bases. However, as shown below, the T_m values of A·T bases can be raised to almost the level of G·C bases. Thus, all A·T or G·C base tetramers (including the ones in white boxes) could potentially be used in a tetramer design. In addition, thymine can be replaced by 5-propynyl uridine when used within capture oligonucleotide address sequences as well as in the oligonucleotide probe addressable array-specific portions. This would increase the T_m of an A·T base pair by ~1.7° C. Thus, T_m values of individual tetramers should be approximately 15.1° C. to 15.7° C. T_m values for the full length 24-mers should be 95° C. or higher.

To illustrate the concept, a subset of six of the 36 tetramer sequences were used to construct arrays: 1=TGCG; 2=ATCG; 3=CAGC; 4=GGTA; 5=GACC; and 6=ACCT. This unique set of tetramers can be used as design modules for the required 24-mer addressable array-specific portion and 24-mer complementary capture oligonucleotide address sequences. This embodiment involves synthesis of five addressable array-specific portion (sequences listed in Table 2). Note that the numbering scheme for tetramers allows abbreviation of each portion (referred to as "Zip #") as a string of six numbers (referred to as "zip code").

TABLE 2

List of all 5 DNA/PNA oligonucleotide address sequences.

Zip #	Zip code	Sequence (5'→3' or NH_2 → COOH)	G + C
Zip11	1-4-3-6-6-1	TGCG-GGTA-CAGC-ACCT-ACCT-TGCG (SEQ. ID. No. 2)	15
Zip12	2-4-4-6-1-1	ATCG-GGTA-GGTA-ACCT-TGCG-TGCG (SEQ. ID. No. 3)	14
Zip13	3-4-5-6-2-1	CAGC-GGTA-GACC-ACCT-ATCG-TGCG (SEQ. ID. No. 4)	15
Zip14	4-4-6-6-3-1	GGTA-GGTA-ACCT-ACCT-CAGC-TGCG (SEQ. ID. No. 5)	14
Zip15	5-4-1-6-4-1	GACC-GGTA-TGCG-ACCT-GGTA-TGCG (SEQ. ID. No. 6)	15

Each of these oligomers contains a hexaethylene oxide linker arm on their 5' termini [P. Grossman, et al., *Nucl. Acids Res.,* 22:4527–4534 (1994), which is hereby incorporated by reference], and ultimate amino-functions suitable for attachment onto the surfaces of glass slides, or alternative materials. Conjugation methods will depend on the free surface functional groups [Y. Zhang, et al., *Nucleic Acids Res.,* 19:3929–3933 (1991) and Z. Guo, et al., *Nucleic Acids Res.,* 34:5456–5465 (1994), which are hereby incorporated by reference].

Synthetic oligonucleotides (normal and complementary directions, either for capture hybridization or hybridization/ligation) are prepared as either DNA or PNA, with either natural bases or nucleotide analogues. Such analogues pair with perfect complementarity to the natural bases but increase T_m values (e.g., 5-propynyl-uracil).

Each of the capture oligonucleotides have substantial sequence differences to minimize any chances of cross-reactivity—see FIG. **17** and Table 1. Rather than carrying out stepwise synthesis to introduce bases one at a time, protected PNA tetramers can be used as building blocks. These are easy to prepare; the corresponding protected oligonucleotide intermediates require additional protection of the internucleotide phosphate linkages. Construction of the 24-mer at any given address requires only six synthetic steps with a likely improvement in overall yield by comparison to stepwise synthesis. This approach eliminates totally the presence of failure sequences on the support, which could occur when monomers are added one-at-a-time to the surface. Hence, in contrast to previous technologies, the possibilities for false signals are reduced. Moreover, since failure sequences at each address are shorter and lacking at least four bases, there is no risk that these will interfere with correct hybridization or lead to incorrect hybridizations. This insight also means that "capping" steps will not be necessary.

Masking technology will allow several addresses to be built up simultaneously, as is explained below. As direct consequences of the manufacturing process for the arrays, several further advantages are noted. Each 24-mer address differs from its nearest 24-mer neighbor by three tetramers, or at least 6 bases. At low salt, each base mismatch in PNA/DNA hybrids decreases the melting temperature by 8° C. Thus, the T_m for the correct PNA/DNA hybridization is at least 48° C. higher than any incorrect hybridization. Also, neighboring 24-mers are separated by 12-mers, which do not hybridize with anything and represent "dead" zones in the detection profile. PNA addresses yield rugged, reusable arrays.

The following description discloses the preparation of 36 unique PNA tetramers and shows the mechanical/chemical strategy to prepare the arrays. This technique can be used to create a 5×5 array with 25 addresses of PNA 24-mers. Alternatively, all 36 tetramers can be incorporated to generate full-size arrays of 1,296 addresses.

FIGS. **18**A–G are schematic diagrams showing addition of PNA tetramers to generate a 5×5 array of unique 24 mer addresses. The manufacturing device is able to add PNA tetramers in either columns, or in rows, by rotating the multi-chamber device or surface 90°. A circular manifold allows circular permutation of tetramer addition. Thus, complex unique addresses may be built by using a simple algorithm. In the first tetramer addition, PNA tetramers **1**, **2**, **3**, **4**, and **5** are linked to the surface in each of the 5 columns, respectively as shown in FIG. **18**A. After rotating the chamber 90°, PNA tetramers **6**, **5**, **4**, **3**, and 2 are added in adjacent rows, as shown in FIG. **18**B. In the third step, as shown in FIG. **18**C, tetramers **3**, **4**, **5**, **6**, and **1** (note circular permutation) are added in columns. In the 4th step, as shown in FIG. **18**D, tetramers **2**, **1**, **6**, **5**, and 4 are added in adjacent rows, etc. This process continues in the manner shown in FIGS. **23**E–G described infra. The bottom of the diagram depicts tetramer sequences which generate unique 24 mers at each position. The middle row of sequences 1-4-3-6-6-1; 2-4-4-6-1-1; 3-4-5-6-2-1; 4-4-6-6-3 and 5-4-1-6-4-1 are shown in full length in Table 2. The addition of tetramers in a circularly permuted fashion can be used to generate larger arrays. Tetramer addition need not be limited to circular patterns and can be added in many other combinations to form unique addresses which differ from each other by at least 3 tetramers, which translates to at least 6 bases.

The present invention has greater specificity than existing mutation detection methods which use allele-specific PCR, differential hybridization, or sequencing-by-hybridization. These methods rely on hybridization alone to distinguish single-base differences in two otherwise identical oligonucleotides. The signal-to-noise ratios for such hybridization are markedly lower than those that can be achieved even with the two most closely-related capture oligonucleotides in an array. Since each address is designed by alternating tetramer addition in three rows and three columns, a given address will differ by at least three tetramers from its neighbor. Since each tetramer differs from every other tetramer by at least 2 bases, a given address will differ from

another address by at least 6 bases. However, in practice, most addresses will differ from most other addresses by considerably more bases.

This concept is illustrated below using the two addresses, Zip 12 and Zip 14. These two addresses are the most related among the 25 addresses schematically represented in FIGS. **18** and **20** (discussed infra). These two addresses have in common tetramers on every alternating position (shown as underlined):

Zip 12 (2-4-4-6-1-1) = 24 mer (SEQ. ID. No. 7)
5'-ATCG GGTA GGTA ACCT TGCG TGCG-3'

Zip 14 (4-4-6-6-3-1) = 24 mer (SEQ. ID. No. 8)
5'-GGTA GGTA ACCT ACCT CAGC TGCG-3'

In addition, they have in common a string of 12 nucleotides, as well as the last four in common (shown as underlined):

Zip 12 (2-4-4-6-1-1) = 24 mer (SEQ. ID. No. 9)
5'-ATCG GGTA GGTA ACCT TGCG TGCG-3'

Zip 14 (4-4-6-6-3-1) = 24 mer (SEQ. ID. No. 10)
5'-GGTA GGTA ACCT ACCT CAGC TGCG-3'

Either representation has at least 8 differences between the oligonucleotides. Although an oligonucleotide complementary to Zip 12 or Zip 14 at the underlined nucleotides could hybridize to both of these addresses at a lower temperature (e.g., 37° C.), only the fully complementary oligonucleotide would hybridize at elevated temperature (e.g., 70° C.).

Furthermore, for other capture oligonucleotides, such as Zip 3, the number of shared nucleotides is much lower (shown as underlined):

Zip 12 (2-4-4-6-1-1) = 24 mer (SEQ. ID. No. 11)
5'-ATCG GGTA GGTA ACCT TGCG TGCG-3'

Zip 3 (3-6-5-2-2-3) = 24 mer (SEQ. ID. No. 12)
5'-CAGC ACCT GACC ATCG ATCG CAGC-3'

Therefore, the ability to discriminate Zip 12 from Zip 3 during hybridization is significantly greater than can be achieved using any of the existing methods.

A multi-chamber device with alternating chambers and walls (each 200 μm thick) will be pressed onto the modified glass or silicon surface of FIG. **19**A prior to delivery of PNA tetramers into either columns or rows. The surface will be etched to produce 10 μm ridges (black lines) to eliminate leaking between chambers. Initially, a flexible spacer (linker) is attached to the array surface. In the first step, as shown in FIG. **19**B, PNA tetramers, **1**, **2**, **3**, **4**, and **5** are linked to the surface in each of the five columns, respectively. The multi-chamber device is then rotated 90°. Tetramers **6**, **5**, **4**, **3**, and **2** are added in adjacent rows, as shown in FIG. **19**C. The process is repeated a total of three times to synthesize 24-mer PNA oligomers. Each completed 24-mer within a given row and column represents a unique PNA sequence, hence achieving the desired addressable array. Smaller oligonucleotide sequences represent half-size 12-mers which result from 3 rounds of synthesis in the same direction. Since each 24-mer differs from its neighbor by three tetramers and each tetramer differs from another by at least 2 bases, then each 24mer differs from the next by at least 6 bases (i.e., 25% of the nucleotides differ). Thus, a wrong address would have 6 mismatches in just 24 bases and, therefore, would not be captured at the wrong address, especially under 75–80° C. hybridization conditions. In addition, while a particular smaller 12-mer sequence may be found within a 24-mer sequence elsewhere on the grid, an addressable array-specific portion will not hybridize to the 12-mer sequence at temperatures above 50° C.

The starting surfaces will contain free amino groups, a non-cleavable amide linkage will connect the C-terminus of PNA to the support, and orthogonal side-chain deprotection must be carried out upon completion of segment condensation assembly in a way that PNA chains are retained at their addresses. A simple masking device has been designed that contains 200 μm spaces and 200 μm barriers, to allow each of 5 tetramers to couple to the solid support in distinct rows (FIG. **20**A). After addition of the first set of tetramers, the masking device is rotated 90°, and a second set of 5 tetramers are added (FIG. **20**B). This can be compared to putting icing on a cake as rows, followed by icing as columns. The intersections between the rows and columns will contain more icing, likewise, each intersection will contain an octamer of unique sequence. Repeating this procedure for a total of 6 cycles generates 25 squares containing unique 24-mers, and the remaining squares containing common 12-mers (FIGS. **20**C and **21**A–F). The silicon or glass surface will contain 10 μm ridges to assure a tight seal, and chambers will be filled under vacuum. A circular manifold (FIG. **26**) will allow for circular permutation of the six tetramers prior to delivery into the five rows (or columns). This design generates unique 24-mers which always differ from each other by at least 3 tetramers, even though some sequences contain the same 3 tetramers in a contiguous sequence. This masking device is conceptually similar to the masking technique disclosed in Southern, et al., *Genomics,* 13:1008–1017 (1992) and Maskos, et al., *Nucleic Acids Res.,* 21:2267–2268 (1993), which are hereby incorporated by reference, with the exception that the array is built with tetramers as opposed to monomers.

Alternatively, the production of the incomplete 12-mer sequences can be eliminated if a mask which isolates each location is used. In the first step (as shown in FIG. **19**D), PNA tetramers **1**, **2**, **3**, **4**, and **5** are linked to the surface in each of the five columns respectively. The multi-chamber device is then rotated 90°. Tetramers **6**, **5**, **4**, **3**, and **2** are added in adjacent rows, as shown in FIG. **19**E. The process is repeated a total of three times to synthesize 24-mer PNA oligomers. Each completed 24-mer within a given row and column represents a unique PNA sequence, hence achieving the desired addressable array. In addition, each 24-mer will be separated from adjacent oligomers by a 200 μm region free of PNA oligomers.

A silicon or glass surface will be photochemically etched to produce a crosshatched grid of 10 μm raised ridges in a checkerboard pattern (see FIG. **20**). Alternate squares (200 μm×200 μm) will be activated, allowing for the attachment of C_{18} alkyl spacers and PEG hydrophilic linkers (MW 400–4,000), such that each square is separated by at least one blank square and two ridges on all sides.

An example of a universal array using PNA tetramers can be formed by adding 36 different tetramers to either 36 columns or rows at the same time. The simplest way to add any tetramer to any row is to have all 36 tetramer solutions attached by tubings to minivalves to a circular manifold which has only one opening. The other side of the circular manifold can be attached to any of 36 minivalves which go to individual rows (or columns). So by rotating the manifold and minivalves to the chambers (rows), one can pump any tetramer into any row, one at a time. This can be either a

mechanical device requiring physical rotation or, alternatively, can be accomplished by using electronic microvalves along a series of import (tetramers) and export (rows) channels. This process can occur quite rapidly (5 seconds, including rinsing out the manifold for its next use), so that it would take about 36×5=180 sec. to add all 36 rows.

A potentially more rapid way of filling the rows or columns, would be to fill all of them simultaneously. This is illustrated in FIG. **20** for a 5×5 array. The silicon or glass surface will contain 10 μm ridges to assure a tight seal, and chambers will be filled using the vacuum technique described above. A circular manifold will allow for circular permutation of the six tetramers prior to delivery into the five rows (or columns). In FIG. **20**, the first step is 5, 4, 3, 2, 1. When rotating the multi-chamber device, one could continue to add in either numerical, or reverse numerical order. In the example, a numerical order of 2, 3, 4, 5, 6 for the second step is used. In the third step, the circular permutation (reverse) gives 1, 6, 5, 4, 3. Fourth step (forward) 4, 5, 6, 1, 2. Fifth step (reverse) 4, 3, 2, 1, 6. Sixth step (forward) 5, 6, 1, 2, 3. This can be expanded to 36 tetramers into 36 rows (or columns). This approach limits the potential variations in making the address for the array from 36^6=2,176,782,336 in every position to 36^6=2,176,782,336 in one position, with the other 1,295 positions now defined by the first address. This is still a vast excess of the number of different addresses needed. Furthermore, each address will still differ from every other address by at least 6 nucleotides.

Note that all of these arrays can be manufactured in groups, just as several silicon chips can be produced on the same wafer. This is especially true of the tetramer concept, because this requires adding the same tetramer in a given row or column. Thus, one row could cover a line of ten arrays, so that a 10×10 grid=100 arrays could be manufactured at one time.

Alternatively, the process described with reference to FIG. **20** can be carried out with one less cycle to make a 20 mer oligonucleotide. The capture oligonucleotide should be sufficiently long to capture its complementary addressable array-specific portion under the selected hybridization conditions.

FIGS. **21**A–F show a schematic cross-sectional view of the synthesis of an addressable array (legend). FIG. **21**A shows attachment of a flexible spacer (linker) to surface of array. FIG. **21**B shows the synthesis of the first rows of oligonucleotide tetramers. Only the first row, containing tetramer **1**, is visible. A multi-chamber device is placed so that additional rows, each containing a different tetramer, are behind the first row. FIG. **21**C shows the synthesis of the first columns of oligonucleotide tetramers. The multi-chamber device or surface has been rotated 90°. Tetramers **9**, **18**, **7**, and **12** were added in adjacent chambers. FIG. **21**D shows the second round synthesis of the oligonucleotide rows. The first row contains tetramer **2**. FIG. **21**E shows the second round of synthesis of oligonucleotides. Tetramers **34**, **11**, **14**, and 23 are added in adjacent chambers during the second round. FIG. **21**F shows the structure of the array after third round synthesis of columns (the first row contains tetramer **3**), adding tetramers **16**, **7**, **20**, **29**. Note that all 24mer oligonucleotides within a given row or column are unique, hence achieving the desired addressable array. Since each 24-mer differs from its neighbor by three tetramers, and tetramers differ from each other by at least 2 bases, then each 24-mer differs from the next by least 6 bases. Each mismatch significantly lowers T_m, and the presence of 6 mismatches in just 24 bases would make cross hybridization unlikely even at 35° C. Note that the smaller 12-mer sequences are identical with one another, but are not at all common with the 24-mer sequences. Even though the particular 12-mer sequence may be found within a 24-mer elsewhere on the grid, for example 17-1-2-3-28-5, an oligonucleotide will not hybridize to the 12-mer at temperatures above 50° C.

FIGS. **22**A–C present a design for a masking device 2 capable of constructing an array grid G as described in FIGS. **19**–**21**. FIG. **22**A is a top view of the arrangement of device 2 and array grid G, while side views FIGS. **22**B–**22**C are, respectively, taken along line **22**B—**22**B and line **22**C—**22**C of FIG. **22**A. The masking device contains 200 μm spaces and 200 μm barriers, to allow each of five tetramers to be coupled to the solid support in distinct rows. After addition of the first set of tetramers, the masking device is rotated 90°, and a second set of 5 tetramers are added. This can be compared to putting icing on a cake as rows, followed by icing as columns. The intersections between the rows and columns will contain more icing, likewise, each intersection will contain an octamer of unique sequence. Repeating this procedure for a total of 6 cycles generates 25 spatially separated squares containing unique 24-mers, and the remaining squares containing common 12-mers. The silicon or glass surface will contain 10 μm ridges R to assure a tight seal, and chambers **4** will be filled by opening valves **8** to a vacuum line **12** to create negative pressure in the chamber. The multi-chamber device is pressed onto the membrane or activated solid surface, forming tight seals. The barriers may be coated with rubber or another material to avoid cross contamination from one chamber to the next. One must also make sure the membrane or solid support surface is properly wetted by the solvents. After closing valves **8** to vacuum line **12**, one proceeds by activating the surface, deprotecting, and adding a tetramer to a chamber **4** through lines **10** by opening valves **6**. The chamber is unclamped, the membrane is rotated 90°, and reclamped. A second round of tetramers are added by the above-described vacuum and tetramer application steps. A valve block assembly (FIGS. **25**A–C) will route each tetramer to the appropriate row. Alternatively, a cylindrical manifold (FIGS. **26**A–D) will allow circular permutation of the six tetramers prior to delivery into the five rows (or columns). This design generates unique 24-mers which always differ from each other by at least 3 tetramers, even though some sequences contain the same 3 tetramers in a contiguous sequence.

FIGS. **23**A–C represent a perspective view of the array construction process described in FIG. **19** (FIGS. **19**D–**19**E). In the first step, as shown in FIG. **23**A, PNA tetramers **1**, **2**, **3**, **4**, and **5** are linked to the surface in each of the five columns, respectively. Each of the 5 locations in the columns are isolated, and there is a 200 μm gap between them where no oligonucleotides are coupled. The multi-chamber device is then rotated 90°, as shown in FIG. **23**B. Tetramers **6**, **5**, **4**, **3**, and **2** are added in adjacent rows. Each of the 5 locations in the rows are isolated, and there is a 200 μm gap between them where no oligonucleotides are coupled. Each completed 24-mer, as shown in FIG. **23**C, within a given row and column represents a unique PNA sequence. Unlike the design presented in FIGS. **19**–**22**, this array design will not contain the half-size 12-mers between each complete 24-mer, because a mask with isolated locations will be used.

FIGS. **24**A–C present a design for a masking device 2 capable of constructing an array grid G as described in FIG. **23**. FIG. **24**A is a top view of the arrangement of device 2 and array grid G, while side views FIGS. **24**B and **24**C are, respectively, taken along line **24**B—24B and line **24**C—24C of FIG. **24**A. The masking device contains 200 μm spaces and 200 μm barriers, to allow each of five tetramers to be

coupled to the solid support in distinct locations on the array grid G. After addition of the first set of tetramers, the masking device or surface is rotated 90°, and a second set of 5 tetramers are added. Repeating this procedure for a total of 6 cycles generates 25 spatially separated squares containing unique 24-mers, and the remaining squares containing common 12-mers. The silicon or glass surface will contain 10 μm ridges R to assure a tight seal, and chambers **4** will be filled by a procedure initiated by using a vacuum to create negative pressure in the chamber **2**. This vacuum is created by opening valves **8** to vacuum line **12**. The multi-chamber device is pressed onto the membrane or activated solid surface, forming tight seals. The barriers may be coated with rubber or another material to avoid cross contamination from one chamber to the next. One must also make sure the membrane or solid support surface is properly wetted by the solvents. After closing valves **8** to vacuum line **12**, one proceeds by activating the surface, deprotecting, and adding a tetramer to chamber **4** through lines **10** by opening valves **6**. The chamber is unclamped, the membrane is rotated 90°, and reclamped. A second round of tetramers are added by the above-described vacuum and tetramer application steps. A valve block assembly (FIGS. **25**A–C) will route each tetramer to the appropriate row. Alternatively, a cylindrical manifold (FIGS. **26**A–D) will allow circular permutation of the six tetramers prior to delivery into the five rows (or columns). This design generates unique 24-mers which are separated from each other by a region free of any oligonucleotides.

FIGS. **25**A–C show a valve block assembly **14** which connects six input ports **10** to five output ports **16** via a common chamber **18**. Each of the **6** input ports **10** and **5** output ports **16** contains a valve **6** and a valve **20**, respectively, which control the flow of fluids. The 6 input tubes **10** contain different solutions, and the valve block assembly **14** is capable of routing any one of the input fluids to one of the 5 output ports **16** at a time. This is accomplished by opening the valve **6** of one of the input ports **10** and one of the valves **20** of the output ports **16** simultaneously and allowing fluid to fill the chamber **18** and exit via the output port **16** connected to the open valve **20**. The valve block assembly **14** is connected to a source of solvent **22** and a source of vacuum **12** via valves **24** and **8**, respectively, in order to allow cleaning of the central chamber **18** in between fluid transfers. The solvent fills the chamber **18**, and the vacuum is used to remove all fluid from the chamber. This prepares the chamber **18** for the next fluid transfer step and prevents cross-contamination of the fluids.

FIGS. **26**A–D depict a cylindrical manifold assembly **114** which transfers 6 different tubes of input fluids to 5 different output tubes. The manifold itself contains two separate halves **114**A and **114**B which are joined by a common, central spoke **134** around which both halves can independently rotate. The bottom portion **114**B is a cylindrical block with 6 channels **130** drilled through it (see FIG. **26**C, which is a bottom view of FIG. **26**B taken along line **26**C—**26**C of FIG. **26**B). Each of the 6 channels **130** are attached to 6 different input tubes **110**. The input tubes **110** contain valves **106** which connect the input channels **130** to either reagents, solvent, or a vacuum via lines **138** having valves **136** leading to vacuum line **112** having valve **108** and solvent line **122** having valve **124**. This allows different fluids to enter the channels **130** and **132** of the manifold and allows clearing of the channels **130** and **132** of excess fluid between fluid transfers. The upper portion of the manifold (see FIG. **26**A, which is a top view of FIG. **26**B taken along line **26**A—**26**A of FIG. **26**B) is also a cylindrical block with 5 channels **132** drilled through it. The 5 channels **132** are each connected to a different output tube **116**. The two halves of manifold **114**A and **114**B can be independently rotated so that different input channels **130** will line up with different output channels **132**. This allows the 6 tubes of input fluids to be transferred to the 5 output tubes simultaneously. The bottom half of the manifold **114**B can be rotated 60 degrees in order to align each input port **110** with the next output port **116**. In this way, each input port **110** can be aligned with any of the output ports **116**. The circular manifold of FIGS. **26**A–D differs from the valve block assembly of FIGS. **26**A–C in that the former can simultaneously transfer five of the six input fluids to the five output ports, because it has 5 channels connecting input ports to the output ports. This concept could be easily expanded to deliver 36 tetramers simultaneously to 36 locations.

The present invention contains a number of advantages over prior art systems.

The solid support containing DNA arrays, in accordance with the present invention, detects sequences by hybridization of ligated product sequences to specific locations on the array so that the position of the signal emanating from captured labels identifies the presence of the sequence. For high throughput detection of specific multiplexed LDR products, addressable array-specific portions guide each LDR product to a designated address on the solid support. While other DNA chip approaches try to distinguish closely related sequences by subtle differences in melting temperatures during solution-to-surface hybridization, the present invention achieves the required specificity prior to hybridization in solution-based LDR reactions. Thus, the present invention allows for the design of arrays of capture oligonucleotides with sequences which are very different from each other. Each LDR product will have a unique addressable array-specific portion, which is captured selectively by a capture oligonucleotide at a specific address on the solid support. When the complementary capture oligonucleotides on the solid support are either modified DNA or PNA, LDR products can be captured at higher temperatures. This provides the added advantages of shorter hybridization times and reduced non-specific binding. As a result, there is improved signal-to-noise ratios.

Another advantage of the present invention is that PCR/LDR allows detection of closely-clustered mutations, single-base changes, and short repeats and deletions. These are not amenable to detection by allele-specific PCR or hybridization.

In accordance with the present invention, false hybridization signals from DNA synthesis errors are avoided. Addresses can be designed so there are very large differences in hybridization T_m values to incorrect address. In contrast, the direct hybridization approaches depend on subtle differences. The present invention also eliminates problems of false data interpretation with gel electrophoresis or capillary electrophoresis resulting from either DNA synthesis errors, band broadening, or false band migration.

The use of a capture oligonucleotide to detect the presence of ligation products, eliminates the need to detect single-base differences in oligonucleotides using differential hybridization. Other existing methods in the prior art relying on allele-specific PCR, differential hybridization, or sequencing-by-hybridization methods must have hybridization conditions optimized individually for each new sequence being analyzed. When attempting to detect multiple mutations simultaneously, it becomes difficult or impossible to optimize hybridization conditions. In contrast, the present invention is a general method for high specificity

detection of correct signal, independent of the target sequence, and under uniform hybridization conditions. The present invention yields a flexible method for discriminating between different oligonucleotide sequences with significantly greater fidelity than by any methods currently available within the prior art.

The array of the present invention will be universal, making it useful for detection of cancer mutations, inherited (germline) mutations, and infectious diseases. Further benefit is obtained from being able to reuse the array, lowering the cost per sample.

The present invention also affords great flexibility in the synthesis of oligonucleotides and their attachment to solid supports. Oligonucleotides can be synthesized off of the solid support and then attached to unique surfaces on the support. Segments of multimers of oligonucleotides, which do not require intermediate backbone protection (e.g., PNA), can be synthesized and linked onto to the solid support. Added benefit is achieved by being able to integrate these synthetic approaches with design of the capture oligonucleotide addresses. Such production of solid supports is amenable to automated manufacture, obviating the need for human intervention and resulting contamination concerns.

An important advantage of the array of the present invention is the ability to reuse it with the previously attached capture oligonucleotides. In order to prepare the solid support for such reuse, the captured oligonucleotides must be removed without removing the linking components connecting the captured oligonucleotides to the solid support. A variety of procedures can be used to achieve this objective. For example, the solid support can be treated in distilled water at 95–100° C., subjected to 0.01 N NaOH at room temperature, contacted with 50% dimethylformamide at 90–95° C., or treated with 50% formamide at 90–95° C. Generally, this procedure can be used to remove captured oligonucleotides in about 5 minutes. These conditions are suitable for disrupting DNA—DNA hybridizations; DNA-PNA hybridizations require other disrupting conditions.

The present invention is illustrated, but not limited, by the following examples.

EXAMPLES

Example 1

Immobilization of capture oligonucleotides to solid supports

The solid support for immobilization was glass, in particular microscope slides The immobilization to glass (e.g., microscope slides), or other supports such as silicon (e.g., chips), membranes (e.g., nylon membranes), beads (e.g., paramagnetic or agarose beads), or plastics supports (e.g., polyethylene sheets) of capture oligonucleotides in spatially addressable arrays is comprised of 5 steps:

A. Silanization of Support

The silanization reagent was 3-aminopropyl triethoxysilane ("APTS"). Alternatively, 3-glycidoxypropyltrimethoxysilane. (K. L. Beattie, et al., "Advances in Genosensor Research," *Clin. Chem.,* 41:700–706 (1995); U. Maskos, et al., "Oligonucleotide Hybridizations on Glass Supports: a Novel Linker for Oligonucleotide Synthesis and Hybridization Properties of Oligonucleotides Synthesized in situ, "*Nucleic Acids Res.,* 20:1679–1684 (1992); C. F. Mandenius, et al., "Coupling of Biomolecules to Silicon Surfaces for Use in Ellipsometry and Other Related Techniques," *Methods Enzymol., pp.* 388–394 (1988), which are hereby incorporated by reference) or 3-(trimethoxysilyl)propyl methacrylate (M. Glad, et al., "Use of Silane Monomers for Molecular Imprinting and Enzyme Entrapment in Polysiloxane-coated Porous Silica," *J. Chromatogr.* 347:11–23 (1985); E. Hedborg, et al., "Some Studies of Molecularly-imprinted Polymer Membranes in Combination with Field-effect Devices," *Sensors and Actuators A* 37–38:796–799 (1993); and M. Kempe, et al., "An Approach Towards Surface Imprinting Using the Enzyme Ribonuclease A," *J. Mol. Recogn.* 8:35–39 (1995), which are hereby incorporated by reference) can be used as an initial silanization reagent. Prior to silanization, the support was cleansed and the surface of the support was rendered hydrophobic. Glass slides (Fisher Scientific, Extra thick microslides, frosted cat.# 12-550-11) were incubated in conc. aq. NH_4OH—H_2O_2—H_2O (1:1:5, v/v/v) at 80° C. for 5 min and rinsed in distilled water. The support was then washed with distilled water, ethanol and acetone as described in the literature (C. F. Mandenius, et al., "Coupling of Biomolecules to Silicon Surfaces for Use in Ellipsometry and Other Related Techniques," *Methods Enzymol*., pp. 388–394 (1988); Graham, et al., "Gene Probe Assays on a Fibre-Optic Evanescent Wave Biosensor," *Biosensors & Bioelectronics,* 7: 487–493 (1992); Jönsson, et al., "Adsorption Behavior of Fibronectin on Well Characterized Silica Surfaces," *J. Colloid Interface Sci.,* 90:148–163 (1982), which are hereby incorporated by reference). The support was silanized overnight at room temperature in a solution of 2% (v/v) 3-aminopropyl triethoxysilane (Sigma, St. Louis, Mo.) in dry acetone (99.7%) (modified after Z. Guo, et. al., "Direct Fluorescence Analysis of Genetic Polymorphisms by Hybridization with Oligonucleotide Arrays on Glass Supports," *Nucl. Acids Res.* 22:5456–65 (1994), which is hereby incorporated by reference). The support was then thoroughly washed in dry acetone and dried at 80° C. in a vacuum desiccator.

B. Derivatization of Silanized Solid Support with Functional Groups (e.g., Carboxyl or Amino Groups)

When the silanization reagent was APTS, the desired amino functionality was introduced directly. Other functional groups can be introduced, either by choosing an appropriate silanization reagent primer that already contains the functional group (e.g., 3-(trimethoxysilyl)propyl methacrylate to functionalize the surface with a polymerizable acrylate, (M. Glad, et al., "Use of Silane Monomers Imprinting and Enzyme Entrapment in Polysiloxane-coated Porous Silica," *J. Chromatogr.* 347:11–23 (1985); E. Hedborg, et al., "Some Studies of Molecularly-imprinted Polymer Membranes in Combination with Field-effect Devices," *Sensors and Actuators A* 37–38:796–799 (1993); and M. Kempe, et al., "An Approach Towards Surface Imprinting Using the Enzyme Ribonuclease A," *J. Mol. Recogn.* 8:35–39 (1995), which are hereby incorporated by reference), or by reacting the amino-functionalized surface with a reagent that contains the desired functional group (e.g., after localized light-directed photodeprotection of protected amino groups used in photolithography, (Fodor, et al., "Light-Directed, Spatially Addressable Parallel Chemical Synthesis," *Science,* 251:767–773 (1991); Fodor, et al., "Multiplexed Biochemical Assays with Biological Chips," *Nature,* 364:555–556 (1993), which are hereby incorporated by reference)).

C. Activation of Functional Groups

The functional group on the solid support was an amino group. Using a prefabricated mask with a 5×5 array of dots that have a diameter of 1 mm, and that are 3.25 mm apart, small amounts (typically 0.2 to 1.0 μl) of a solution containing 70 mg/ml disuccinimidyl adipate ester (Hill, et al.,

"Disuccinimidyl Esters as Bifunctional Crosslinking Reagents for Proteins," *FEBS Lett,* 102:282–286 (1979); Horton, et al., "Covalent Immobilization of Proteins by Techniques which Permit Subsequent Release," *Methods Enzmmol., pp.* 130–141 (1987), which are hereby incorporated by reference) in anhydrous dimethylformamide ("DMF"); Aldrich, Milwaukee, Wis.), amended with 1–2% triethylamine (to scavenge the acid that is generated), were manually applied to the solid support using a Gilson P-10 pipette. After application, the reaction was allowed to proceed for 30 min at room temperature in a hood, after which another loading of disuccinimidyl adipate ester was applied. After a total reaction time of 1 hour, the support was washed with anhydrous DMF and dried at room temperature in a vacuum desiccator.

In case the functional group is a carboxyl group, the solid support can be reacted with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride ("EDC"). Frank, et al., "Simultaneous Multiple Peptide Synthesis Under Continuous Flow Conditions on Cellulose Paper Discs as Segmental Solid Support," *Tetrahedron,* 44:6031–6040 (1988), which is hereby incorporated by reference. Prior to this reaction, the surface of the solid support was protonated by a brief treatment with 0.1 N HCl. Using the above described prefabricated mask, small amounts (0.2 to 1.0 μl) of a fresh solution containing 1 M EDC (Sigma, St. Louis, Mo.), 1 mM of 5' amino-modified oligonucleotide and 20 mM KH_2PO_4, pH=8.3, was manually applied to the solid support. The reaction was allowed to proceed for 1 hour, after which the support was washed with distilled water and dried at room temperature in a vacuum desiccator.

D. Coupling of Amino-Functionalized Capture Oligonucleotides to the Preactivated Solid Support

For supports other than EDC-activated solid supports, small amounts (0.2 to 1.0 μl) of 1 nmol/μl 5' amino-modified oligonucleotides (i.e. the sequences in Table 2) in 20 mM KH_2PO_4, pH 8.3, were manually applied to the activated support, again using the prefabricated mask described above. The reaction was allowed to proceed for 1 hour at room temperature.

E. Quenching of Remaining Reactive Groups on the Solid Support

In order to prevent the reaction products from being nonspecifically captured on the solid support in a capture probe-independent way, it may be necessary to quench any remaining reactive groups on the surface of the solid support after capture of the complementary oligonucleotide probes. Hereto, the support was incubated for 5 min at room temperature in 0.1 N sodium hydroxide. Alternatively, quenching can be performed in 0.2 M lysine, pH=9.0. After quenching, the support was washed with 0.1 N sodium phosphate buffer, pH 7.2, to neutralize the surface of the support. After a final wash in distilled water the support was dried and stored at room temperature in a vacuum desiccator.

Example 2

Design of the Assay System

A semi-automated custom-designed assay system was made for testing hybridizations and subsequent washings of captured oligonucleotide probe-capture oligonucleotide hybrids in a high-throughput format using the GeneAmp In Situ PCR System 1000™ (Perkin Elmer, Applied Biosystems Division, Foster City, Calif.) (G. J. Nuovo, *PCR in situ Hybridization*, New York: Raven Press (2nd ed. 1994), which is hereby incorporated by reference). A general flowchart of the system is shown in FIG. **27**. The system consists of a flow-through hybridization chamber which is connected via a sample loading device and a multiple port system to a battery of liquid reservoirs, and to a waste reservoir. The fluid delivery is controlled by a pump. The pump was placed at the end of the assembly line and operated under conditions to maintain a light vacuum to prevent leakage and contamination of the system. Since the hybridization chamber and the liquid reservoirs were designed to fit precisely within the GeneAmp In Situ PCR System 1000™, temperatures can be accurately controlled and maintained during the hybridization and washing steps of the assay.

The individual parts of the system are described in detail in the following section:

A. Hybridization Chamber

The hybridization chamber is an in situ PCR reagent containment system that has been modified to accommodate flow-through characteristics. The containment system is comprised of a glass microscope slide (76×25×1.2±0.02 mm) and a silicone rubber diaphragm, which has been clamped to the slide by a thin stainless steel clip. The inside oval rim of the metal clip compresses the edges of the silicon disc against the slide with enough force to create a water and gas-tight seal ensuring the containment of hybridization probes and washing liquids. The volume of the containment is approximately 50 μl. The array of immobilized capture oligonucleotides is contained in the central area of the slide (approximately 13 mm×15 mm) which is covered by the silicon disc. The assembly of the different parts is facilitated by an assembly tool which is provided by the manufacturer of the in situ PCR system. Once assembled, an inlet and outlet of the hybridization chamber is created by insertion of two 25G¾ needles with 12" tubing and multiple sample luer adapter (Becton Dickinson, Rutherford, N.J.). The needles are inserted in a diagonal manner to assure an up-and-across flow pattern during washing of the probe-target hybrids.

B. Liquid Reservoirs

Reservoirs containing different washing solutions were custom-designed to fit into the vertical slots of the thermal block of the GeneAmp In Situ PCR System 1000™. Each reservoir consists of two glass microscope chamber slides (25×75×1 mm) containing prefabricated silicone gaskets (Nunc, Inc., Napierville, Ill.), which were glued to each other using silicone sealant (Dow Corning, Midland, Mich.). An outlet was created by insertion of a 21G ¾" needle with 12" long tubing and multiple sample luer adapter (Becton Dickinson, Rutherford, N.J.) through the silicone gasket. A second 21 G ¾" needle without tubing (Becton Dickinson, Franklin Lakes, N.J.) was inserted through the silicone gasket to create an air inlet. The liquid reservoirs are leak-free and fit precisely within the slots of the thermal block, where they are clamped against the metal fins to assure good heat transfer to the contained liquid. The volume of each reservoir is approximately 2 ml.

C. Multi Port System and Sample Loading Device

Liquid reservoirs, sample loading device and hybridization chamber are connected through a multiple port system that enables a manually controlled unidirectional flow of liquids. The system consists of a series of 3-way nylon stopcocks with luer adapters (Kontes Scientific Glassware/Instruments, Vineland, N.J.) that are connected to each other through male-female connections. The female luer adapters from the liquid reservoirs are connected to the multi port female luer adapters via a male-to-male luer adapter coupler (Biorad, Richmond, Calif.). The sample loading device is placed in between the ports connected to the liquid reservoirs and the port connected to the hybridization chamber. It

consists of a 1 ml syringe (Becton Dickinson, Franklin Lakes, N.J.) that is directly connected via a luer adapter to the multi port system. The flow of liquids can be controlled manually by turning the handles on the stopcocks in the desired direction.

D. Waste Reservoir

The outlet tubing from the hybridization chamber is connected to a waste reservoir which consists of a 20 ml syringe with luer adapter (Becton Dickinson, Franklin Lakes, N.J.) in which the plunger has been secured at a fixed position. A connection to the pump is established by insertion of a 21G ¾" needle with 12" long tubing and multiple sample luer adapter through the rubber gasket of the plunger. When the pump is activated, a slight vacuum is built up in the syringe which drives the flow of liquids from the liquid reservoirs through the hybridization chamber to the waste reservoir.

E. Pump

A peristaltic pump P-1 (Pharmacia, Piscataway, N.J.) was used to control the flow of liquids through the system. It was placed at the end of the assembly line in order to maintain a slight vacuum within the system. The inlet tubing of the pump was connected to the outlet tubing of the waste reservoir via a 3-way nylon stopcock. By this construction release of the vacuum within the waste reservoir is established enabling its draining by gravity.

Example 3

Hybridization and Washing Conditions

In order to assess the capture specificity of different capture oligonucleotides, hybridization experiments were carried out using two capture oligonucelotide probes that had 3 out of 6 tetramers (i.e., 12 out of 24 nucleotides) in common. This example represents the most difficult case to distinguish between different capture oligonucleotides. In general, other capture oligonucleotides would be selected that would have fewer tetramers in common to separate different amplification products on an addressable array.

Typically, 10 pmol of each of the oligonucleotides comp 12 and comp 14 (see Table 3) were 5' end labeled in a volume of 20 μl containing 10 units of T4 polynucleotide kinase (New England Biolabs, Beverly, Mass.), 2.22 MBq (60 μCi) [γ-^{32}P] ATP, 50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 1 mM EDTA, and 10 mM dithiothreitol, according to a slightly modified standard procedure described in the literature. Unincorporated radioactive nucleotides were removed by filtration over a column containing superfine DNA grade Sephadex G-25 (Pharmacia, Piscataway, N.J.). The Sephadex was preswollen overnight at 4° C. in 10 mM ammonium acetate. The labeled oligonucleotide probes were dried in vacuum and dissolved in hybridization solution (0.5 M Na_2HPO_4 [pH 7.2], 1% crystalline grade BSA, 1 mM EDTA, 7% SDS). The specific activity of the labeled oligonucleotide probes comp 12 and comp 14 was 2.86×10^6 cpm/pmol and 2.43×10^6 cpm/pmol, respectively.

Four hundred picomoles of amino-linked capture oligonucleotides 12 and 14 (see Table 3) were deposited and reacted both on carboxyl derivatized and amino derivatized glass microscope slides as described in the previous section. The capture oligonucleotides were immobilized in a 2×2 matrix array, in such a way that hybridization with the complementary oligonucleotide probe comp 12 would result in a positive signal for the top-left and bottom-right diagonal positions, while hybridization with the complementary oligonucleotide probe comp 14 would result in a positive signal for the bottom-left and top-right diagonal positions.

Radiolabeled oligonucleotide probes comp 12 and comp 14 (see Table 3) were dissolved in hybridization solution at a concentration of 2.5 pmol/100 μl and 4.1 pmol/100 μl, respectively. The hybridization solutions were amended with 5 μl of a 2% bromophenol blue marker to facilitate the visual monitoring of the probes during their transport through the assay system. One hundred microliters of radiolabeled probe was then injected and pumped into the hybridization chamber. Hybridizations were performed for 15 min at 70° C.

After hybridization, the hybridization chamber was sequentially washed with 2×2 ml of low stringency wash buffer (2× SSC buffer contains 300 mM sodium chloride and 30 mM sodium citrate), 0.1% sodium dodecylsulfate ("SDS")) and 2×2 ml of high stringency wash buffer (0.2× SSC, 0.1% SDS) at 70° C. (1× SSC buffer contains 150 mM sodium chloride and 15 mM sodium citrate).

Example 4

Detection of Captured Oligonucleotide Probes

After washing the capture oligonucleotide—oligonucleotide probe hybrids, silicon discs, needles and metal cover clips were removed from the glass microscope slides, and remaining liquid was absorbed using Kimwipes (Kimberly-Clark, Roswell, Ga.). The captured oligonucleotide probes were visualized and quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, Calif.). After 21 hours of exposure of the glass microscope slide to a phosphorimager screen, data were collected for the different solid supports that were tested. The images that were obtained are shown in FIG. **28**. Quantitative data are shown in Tables 4A and 4B.

Under the conditions that were used, the signals and cross-reactivity data that were obtained for the NH_2-functionalized slides were better than those obtained for the COOH-functionalized slides.

TABLE 3

Oligonucleotides used (5' to 3')		
12	Aminolink- spacer 18-	ATC GGG TAG GTA ACC TTG CGT GCG (SEQ. ID. No. 13)
14	Aminolink- spacer 18-	GGT AGG TAA CCT ACC TCA GCT GCG (SEQ. ID. No. 14)
comp 12		CGC ACG CAA GGT TAC CTA CCC GAT (SEQ. ID. No. 15)
comp 14		CGC AGC TGA GGT AGG TTA CCT ACC (SEQ. ID. No. 16)

TABLE 4A

Quantification of captured oligonucleotide probe 12

Functional group		Oligonucleotide probe at capture oligonucleotide 12		Oligonucleotide probe at capture oligonucleotide 14		Average cross
on slide	Probe	(pic)*	(amol)	(pic)*	(amol)	reactivity
—COOH	12	105,333	9.0			0.37
—COOH	12	55,957	4.8			
—COOH	12			36,534	3.1	
—COOH	12			23,707	2.0	
—NH_2	12	353,569	30			0.015
—NH_2	12	10,421,092	889			
—NH_2	12			64,999	5.5	
—NH_2	12			95,414	8.1	

*pic = relative phosphorimager counts

TABLE 4B

Quantification of captured oligonucleotide probe 14

Functional group		Oligonucleotide probe at capture oligonucleotide 12		Oligonucleotide probe at capture oligonucleotide 14		Average cross
on slide	Probe	(pic)*	(amol)	(pic)*	(amol)	reactivity
—COOH	14			35,610	4.0	0.19
—COOH	14			43,362	4.9	
—COOH	14	5,587	0.6			
—COOH	14	9,379	1.1			
—NH_2	14			245,973	28	0.049
—NH_2	14			115,529	13	
—NH_2	14	9,775	1.1			
—NH_2	14	8,065	0.9			

*pic = relative phosphorimager counts

Example 5

Optimizing Immobilization Parameters of Capture Oligonucleotides

Polymer was deposited on slides using a literature procedure. Barnard, et al., "A Fibre-optic Sensor With Discrete Sensing Sites," *Nature* 353:338–40 (1991); Bonk, et al., "Fabrication of Patterned Sensor Arrays With Aryl Azides on a Polymer-coated Imaging Optical Fiber Bundle," *Anal. Chem.* 66:3319–20 (1994); Smith, et al., "Poly-N-acrylylpyrrolidone—A New Resin in Peptide Chemistry," *Int. J. Peptide Protein Res.* 13:109–12 (1979), which are hereby incorporated by reference.

Four hundred picomoles of amino-linked capture oligonucleotides 12 and 14 (see Table 3) were deposited and reacted in a 2×2 pattern to a glass microscope slide that contained 4 identical photo-deposited polymer spots. The oligonucleotides were spotted in such a way that hybridization with the complementary oligonucleotide probe comp 12 would result in a positive signal for the top and bottom positions, while hybridization with the complementary oligonucleotide probe comp 14 would result in a positive signal for the left and right positions.

Radiolabeled oligonucleotide probe comp 12 (see Table 3) was dissolved in hybridization solution at a concentration of 2.4 pmol/100 μl. Bromophenol blue marker (5 μl of a 2% solution) was added to the hybridization solution to facilitate the monitoring of the probe during its transport through the system.

One hundred microliters of radiolabeled probe comp 12 was pumped into the hybridization chamber. Hybridization was performed for 15 min at 70° C. After hybridization, the hybridization chamber was sequentially washed with 3×1 ml of low stringency wash buffer (2× SSC, 0.1% SDS) and 3×1 ml of high stringency wash buffer (0.2× SSC, 0.1% SDS) at 70° C.

After 24 hours of exposure of the glass microscope slide to a phosphorimager screen, data were collected for all the different slides that were tested. The images that were obtained are shown in FIG. **29**. Quantitative data are shown in Table 5.

TABLE 5

Quantification of captured oligonucleotide probes

Crosslinker	Percentage crosslinker	probe 12 (pic)*	probe 12 (amol)
EGDMA	2	1,055,100	80
		1,390,499	106
HDDMA	2	633,208	48
		286,9371	218
EGDMA	4	4,449,001	338
		2,778,414	211

EGDMA = ethylene glycol dimethacrylate
HDDMA = hexane diol dimethacrylate
*pic = relative phosphorimager counts

The immobilization chemistry allows for the use of tailor-made specialty polymer matrices that provide the appropriate physical properties that are required for efficient capture of nucleic acid amplification products. The specificity of the immobilized capture oligonucleotides has been relatively good compared to current strategies in which single mis-

matches, deletions, and insertions are distinguished by differential hybridization (K. L. Beattie, et. al. "Advances in Genosensor Research," *Clin. Chem.* 41:700–06 (1995), which is hereby incorporated by reference). Finally, it has been demonstrated that the assay system of the present invention enables the universal identification of nucleic acid oligomers.

Example 6

Capture of Addressable Oligonucleotide Probes to Solid Support

Polymer-coated slides were tested for their capture capacity of addressable oligonucleotide probes following different procedures for immobilization of capture oligonucleotides. After being silanized with 3-(trimethoxysilyl) propyl methacrylate, monomers were then polymerized on the slides. In one case, a polymer layer having COOH functional groups was formed with a polyethylene glycol-containing crosslinker. In the other case, a polyethylene glycol-methacrylate monomer was polymerized onto the slide to form OH functional groups. The slides with the COOH functional groups were activated using the EDC-activation procedure of Example 1.

The slide with OH functional groups was activated overnight at room temperature by incubation in a tightly closed 50 ml plastic disposable tube (Corning Inc., Corning, N.Y.) containing 0.2 M 1,1'-carbonyldiimidazole ("CDI") (Sigma Chemical Co., St. Louis, Mo.) in "low water" acetone (J. T. Baker, Phillipsburg, N.J.). The slide was then washed with "low water" acetone, and dried in vacuum at room temperature.

Amino-linked capture oligonucleotide 14 was manually spotted on premarked locations on both sides (4 dots per slide). The reactions were performed in a hood, and the amount of oligonucleotide that was spotted was 2×0.2 μl (0.8 mmol/μl). The total reaction time was 1 hr. The slides were then quenched for 15 min by the application of few drops of propylamine on each of the premarked dots. After quenching, the slides were incubated for 5 min in 0.1 N sodium phosphate buffer, pH 7.2, washed in double distilled H_2O, and dried in vacuum.

The complementary capture oligonucleotides on the slides were hybridized with radioactively labeled oligonucleotide probe comp 14 (Table 3). One hundred microliters radiolabeled oligonucleotide probe comp 14 (2.8 pmol; 6,440,000 cpm/pmol) were pumped into the hybridization chamber. Hybridization was performed for 15 min at 70° C. in 0.5 M Na_2HPO_4 [pH 7.2], 1% crystalline grade BSA, 1 mM EDTA, 7% SDS. After hybridization, the hybridization chamber was sequentially washed with 2×2 ml of low stringency wash buffer (2× SSC, 0.1% SDS) and 2×2 ml of high stringency wash buffer (0.2× SSC, 0.1% SDS) at 70° C. (1 SSC buffer contains 150 mM sodium chloride and 15 mM sodium citrate).

After 30 min of exposure of the glass microscope slide to a phosphorimager screen, data were collected for both slides. After 30 minutes of exposure of the glass microscope slides to a phosphorimager screen data were collected. See Table 6 and FIG. **30**.

TABLE 6

Quantification of capture oligonucleotide probe 14 on OH-functionalized slides

Functional group		Oligonucleotide probe at capture oligonucleotide 14	
on glass slide	Probe	(pic)*	(fmol)
—OH	14	1,864,879	10.9
—OH	14	1,769,403	10.3

*pic = relative phosphorimager counts

In this test, better results were obtained with the slide coated with the polymer containing OH functional groups than with the slide coated with the polymer containing COOH functional groups.

With previously prepared (poly HEMA)-containing polymers that were polymerized with 20% amine-containing monomers and crosslinked with 4% EGDMA or HDDMA, it was possible to capture about 275 amol of radioactively labelled ligated product sequence (which could only be visualized after 23 hours of exposure to a phosphorimager screen (Table 5)). Using the polyethylene-methacrylate polymer formulations, it was possible to capture about 10.6 fmoles of ligated product sequence. The signal could be detected after 30 min of exposure.

Example 7

Detection of Captured Oligonucleotides Using a Membrane Support

In order to assess the capture specificity of different capture oligonucleotides using a membrane support, hybridization experiments were carried out using the capture oligonucleotide probes 12 and 14 (Table 3).

Strips of OH-functionalized nylon membrane (Millipore, Bedford, Mass.) were soaked overnight in a 0.2 M solution of carbonyldiimidazole in "low water" acetone. The strips were washed in acetone and dried in vacuo. Two volumes of 0.2 μl (1 mM) capture oligonucleotides 12 and 14 in 20 mM K_2HPO_4, pH 8.3, (Table 3) were loaded on the membrane using a special blotting device (Immunetics, Cambridge, Mass.). Complementary oligonucleotide probes were radioactively labeled as described in Example 3. The oligonucleotide probes were dried in vacuo and taken up in 200 μl hybridization buffer (0.5 M Na_2HPO_4 [pH 7.2], 1% crystalline grade BSA, 1 mM EDTA, 7% SDS). Membranes were prehybridized in 800 μl hybridization buffer for 15 min at 60° C. in 1.5m1 Eppendorf tubes in a Hybaid hybridization oven. The tubes were filled with 500 μl of inert carnauba wax (Strahl & Pitsch, Inc., New York, N.Y.) to reduce the total volume of the hybridization compartment. After prehybridization, 200 μl of radiolabeled probe was added. The membranes were hybridized for 15 min at 60° C. After hybridization, the membranes were washed at 60° C., twice for 15 min with 1 ml of low stringency wash buffer (2× SSC, 0.1% SDS), and twice for 15 min with 1 ml of high stringency wash buffer (0.2× SSC, 0.1% SDS). The captured oligonucleotide probes were quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, Calif.). After 45 min of exposure to a phosphorimager screen, data were collected. The results are shown in Table 7, where the activities of capture oligonucleotides 12 and 14 are 112 pic/amol and 210 pic/amol, respectively.

TABLE 7

Functional group		Quantification of captured oligonucleotides on membranes: Oligonucleotide probe at capture oligonucleotide 12		Oligonucleotide probe at capture oligonucleotide 14		Average cross
on membrane	Probe	(pic)*	(fmol)	(pic)*	(fmol)	reactivity
—OH	12	13,388,487	119.5	337,235	3.01	0.025
—OH	12	13,299,298	118.7			
—OH	14	179,345	0.85	1,989,876	9.48	0.071
—OH	14			3,063,387	14.59	

*pic = relative phosphorimager counts

Hybridization temperatures and hybridization times were further explored in a series of similar experiments. The data shown in Table 8 (where the activities of capture oligonucleotides 12 and 14 are 251 pic/amol and 268 pic/amol, respectively) represent the results obtained with the following conditions: 15 min prehybridization at 65° C. in 800 μl hybridization buffer; 15 min hybridization at 65° C. in 1 ml hybridization buffer; 2× washings for 5 min at 65° C. with 1 ml of low stringency wash buffer; and 2× washings for 5 min at 65° C. with 1 ml of high stringency wash buffer.

TABLE 8

Functional group		Quantification of captured oligonucleotides on membranes: Oligonucleotide probe at capture oligonucleotide 12		Oligonucleotide probe at capture oligonucleotide 14		Average cross
on membrane	Probe	(pic)*	(fmol)	(pic)*	(fmol)	reactivity
—OH	12	41,023,467	163.4	541,483	2.16	0.015
—OH	12	31,868,432	127.0			
—OH	14	294,426	1.10	19,673,325	73.41	0.016
—OH	14			18,302,187	68.29	

*pic = relative phosphorimager counts

The data shown in Table 9 (where the activities of capture oligonucleotides 12 and 14 are 487 pic/amol and 506 pic/amol, respectively) represent the results obtained with the following conditions: 15 min prehybridization at 70° C. in 150 μl hybridization buffer; 15 min hybridization at 70° C. in 200 μl hybridization buffer; 2× washings for 5 min at 70° C. in 800 μl of low stringency wash buffer; and 2× washings for 5 min at 70° C. in 800 μl of high stringency wash buffer.

TABLE 9

Functional group		Quantification of captured oligonucleotides on membranes: Oligonucleotide probe at capture oligonucleotide 12		Oligonucleotide probe at capture oligonucleotide 14		Average cross
on membrane	Probe	(pic)*	(fmol)	(pic)*	(fmol)	reactivity
—OH	12	34,648,385	71.15	1,158,832	2.38	0.027
—OH	12	52,243,549	107.28			
—OH	14	1,441,691	2.85	56,762,990	112.18	0.028
—OH	14			45,769,158	90.45	

*pic = relative phosphorimager counts

The data shown in Table 10 represent the results obtained with the following conditions: 15 min prehybridization at 70° C. in 150 μl hybridization buffer; 5 min hybridization at 70° C. in 200 μl hybridization buffer; 2× washings for 5 min at 70° C. with 800 μl of low stringency wash buffer; and 2× washings for 5 min at 70° C. with 800 μl of high stringency wash buffer.

TABLE 10

Quantification of captured oligonucleotides on membranes.

Functional group		Oligonucleotide probe at capture oligonucleotide 12		Oligonucleotide probe at capture oligonucleotide 14		Average cross
on membrane	Probe	(pic)*	(fmol)	(pic)*	(fmol)	reactivity
—OH	12	26,286,188	53.98	389,480	0.80	0.013
—OH	12	34,879,649	71.62			
—OH	14	539,486	1.07	45,197,674	89.32	0.011
—OH	14			54,409,947	107.53	

*pic = relative phosphorimager counts

The data shown in Table 11 represent the results obtained with the following conditions: 5 min prehybridization at 70° C. in 150 μl hybridization buffer; 1 min hybridization at 70° C. in 200 μl hybridization buffer; 2× washings for 2 min at 70° C. with 800 μl of low stringency wash buffer; and 2× washings for 5 min at 70° C. with 800 μl of high stringency wash buffer.

TABLE 11

Quantification of captured oligonucleotides on membranes

Functional group		Oligonucleotide probe at capture oligonucleotide 12		Oligonucleotide probe at capture oligonucleotide 14		Average cross
on membrane	Probe	(pic)*	(fmol)	(pic)*	(fmol)	reactivity
—OH	12	5,032,835	10.33	56,777	0.12	0.012
—OH	12	4,569,483	9.38			
—OH	14	540,166	1.07	41,988,355	82.98	0.017
—OH	14			20,357,554	40.23	

*pic = relative phosphorimager counts

These data demonstrate that hybridization of the capture oligonucleotide probes to their complementary sequences was specific. In comparison with the previous experiments performed with glass slides, significantly greater amounts (i.e., fmol quantities compared to amol quantities) of oligonucleotide probes were reproducibly captured on the membrane supports. For these two very closely-related capture oligonucleotide probes, average cross-reactivity values of about 1% could be obtained. However, for other pairs of capture oligonucleotides in the array, these values would be significantly better. In general, such values cannot be achieved by using existing methods that are known in the art, i.e., by allele-specific oligonucleotide hybridization ("ASO") or by differential hybridization methods, such as sequencing by hybridization ("SBH").

Example 8

Cleaning Glass Surfaces

Glass slides (Fisher Scientific, Extra thick microslides, frosted cat.# 12–550-11) were incubated in conc. aq. $NH_4OH—H_2O_2—H_2O$ (1:1:5, v/v/v) at 80° C. for 5 min and rinsed in distilled water. A second incubation was performed in conc. aq $HCl-H_2O_2—H_2O$ (1:1:5,v/v/v) at 80° C. for 5 min. See U. Jonsson, et al., "Absorption Behavior of Fibronectin on Well Characterized Silica Surfaces," *J. Colloid Interface Sci.* 90:148–163 (1982), which is hereby incorporated by reference. The slides were rinsed thoroughly in distilled water, methanol, and acetone, and were air-dried at room temperature.

Example 9

Silanization with 3-methacryloyloxypropyltrimethoxysilane

Cleaned slides, prepared according to Example 8, were incubated for 24–48 h at room temperature in a solution consisting of 2.6 ml of 3-methacryloyloxypropyltrimethoxysilane (Aldrich Chemical Company, Inc. Milwaukee, Wis. cat.# 23,579–2), 0.26 ml of triethylamine, and 130 ml of toluene. See E. Hedborg, et. al., *Sensors Actuators A,* 37–38:796–799 (1993), which is hereby incorporated by reference. The slides were rinsed thoroughly in acetone, methanol, distilled water, methanol again, and acetone again, and were air-dried at room temperature. See FIG. **31**.

Example 10

Silanization with Dichlorodimethylsilane

Cleaned slides, prepared according to Example 8, were incubated for 15 min at room temperature in a solution containing 12 ml of dichlorodimethylsilane and 120 ml of toluene. The slides were rinsed thoroughly in acetone, methanol, distilled water, methanol again, and acetone again and were air-dried.

Example 11

Polymerization of Poly(ethylene glycol)methacrylate with Methacrylate-derivatized Glass

2.2 g of poly(ethylene glycol)methacrylate (Aldrich Chemical Company, Inc. Milwaukee, Wis. cat.# 40,953–7) (average M~306 g/mol) and 50 mg of 2,2'-azobis(2-methylpropionitrile) in 3.5 ml of acetonitrile were cooled on ice and purged with a stream of argon for 3 min. The next steps were performed in a glovebox under argon atmosphere. 5–15 drops of the polymerization mixture were placed on a methacrylate-derivatized glass slide, prepared according to Examples 8 and 9. The methacrylate-derivatized glass slide and the polymerization mixture were covered by a second glass slide which had been silanized according to Example 10, and the two glass slides were pressed together and fixed with clips. The slides were subsequently transferred to a vacuum desiccator. The polymerization was thermolytically initiated at 55° C., or photolytically at 366 nm. See FIG. **32**.

Example 12

Polymerization of Acrylic Acid and Trimethylolpropane Ethoxylate (14/3 EO/OH) Triacrylate with Methacrylate-Derivatized Glass

0.5 g of acrylic acid (Aldrich Chemical Company, Inc. Milwaukee, Wis. cat.# 14,723-0), 1.83 g of trimethylolpropane ethoxylate (14/3 EO/OH) triacrylate (Aldrich Chemical Company, Inc. Milwaukee, Wis. cat.# 23,579-2) and 50 mg of 2,2'-azobis(2-methylpropionitrile) in 3.5 ml of acetonitrile were cooled on ice and purged with a stream of argon for 3 min. The next steps were performed in a glovebox as described in Example 11. The slides were subsequently transferred to a vacuum desiccator and polymerized as described in Example 11. See FIG. **33**.

Example 13

Polymerization of Poly(ethylene glycol)methacrylate and Trimethylolpropane Ethoxylate (14/3 EO/OH) Triacrylate with Methacrylate-derivatized Glass

0.55 g of poly(ethylene glycol)methacrylate (Aldrich Chemical Company, Inc. Milwaukee, Wis. cat.# 40,953,7), 1.64 g of trimethylolpropane ethoxylate (14/3 EO/OH triacrylate (Aldrich Chemical Company, Inc. Milwaukee, Wis. cat.# 23,579-2), and 50 mg of 2,2'-azobis(2-methylpropionitrile) in 3.5 ml of acetonitrile were cooled on ice and purged with a stream of argon for 3 min. The next steps were performed in a glove-box as described in Example 11. The slides were subsequently transferred to a vacuum desiccator and polymerized as described in Example 11. See FIG. **34**.

Although the invention has been described in detail for the purpose of illustration, it is understood that such details are solely for that purpose and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

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<223> OTHER INFORMATION: Description of Artificial Sequence: Oligonucleotide

<400> SEQUENCE: 16

cgcagctgag gtaggttacc tacc 24

What is claimed:

1. A device comprising:

a solid support having an array of positions each suitable for attachment of an oligonucleotide probe;

a linker suitable for coupling an oligonucleotide probe to the solid support and attached to the solid support at each of the array positions; and

an array of capture oligonucleotide probes on the solid support at the array positions, said capture oligonucleotide probes each having greater than sixteen nucleotides and able to bind to complementary nucleic acids at uniform hybridization conditions, wherein each capture oligonucleotide probe of the array differs in sequence from its adjacent capture oligonucleotide probe, when aligned to each other by at least 25% of the nucleotides.

2. The device according to claim **1**, wherein different capture oligonucleotide probes are attached at different array positions on the solid support to detect different nucleic acids.

3. The device according to claim **1**, wherein the solid support is made from a material selected from the group consisting of plastic, ceramic, metal, resin, gel, glass, silicon, and composites thereof.

4. The device according to claim **1**, wherein the solid support is in a form selected from the group consisting of slides, discs, membranes, films, and composites thereof.

5. The device according to claim **1**, wherein the solid support has an array of positions with capture oligonucleotide probes attached to all of the array of positions.

6. The device according to claim **5**, wherein the solid support has wells, raised regions, or etched trenches.

7. The device according to claim **6**, wherein the solid support is in a microtiter plate.

8. The device according to claim **1**, wherein the linker comprises a silane on a surface of the solid support.

9. The device according to claim **1**, wherein the solid support is functionalized with olefin, amino, hydroxyl, silanol, aldehyde, keto, halo, acyl halide, or carboxyl groups.

10. The device according to claim **9**, wherein the solid support is functionalized with an amino group by reaction with an amine compound selected from the group consisting of 3-aminopropyl triethoxysilane, 3-aminopropylmethyldiethoxysilane, 3-aminopropyl dimethylethoxysilane, 3-aminopropyl trimethoxysilane, N-(2-aminoethyl)-3-aminopropylmethyl dimethoxysilane, N-(2-aminoethyl-3-aminopropyl) trimethoxysilane, aminophenyl trimethoxysilane, 4-aminobutyldimethyl methoxysilane, 4-aminobutyl triethoxysilane, aminoethylaminomethylphenethyl trimethoxysilane, and mixtures thereof.

11. The device according to claim **9**, wherein the solid support is functionalized with an olefin-containing silane.

12. The device according to claim **11**, wherein the olefin-containing silane is selected from the group consisting of 3-(trimethoxysilyl)propyl methacrylate, N-[3-(trimethoxysilyl)propyl]-N'-(4-vinylbenzyl)ethylenediamine, triethoxyvinylsilane, triethylvinylsilane, vinyltrichlorosilane, vinyltrimethoxysilane, vinyltrimethylsilane, and mixtures thereof.

13. The device according to claim **11**, wherein the silanized support is polymerized with an olefin containing monomer.

14. The device according to claim **13**, wherein the olefin-containing monomer contains a functional group.

15. The device according to claim **14**, wherein the olefin-containing monomer is selected from the group consisting of acrylic acid, methacrylic acid, vinylacetic acid, 4-vinylbenzoic acid, itaconic acid, allyl amine, allylethylamine, 4-aminostyrene, 2-aminoethyl methacrylate, acryloyl chloride, methacryloyl chloride, chlorostyrene, dichlorostyrene, 4-hydroxystyrene, hydroxymethylstyrene, vinylbenzyl alcohol, allyl alcohol, 2-hydroxyethyl methacrylate, poly(ethylene glycol) methacrylate, and mixtures thereof.

16. The device according to claim **13**, wherein the support is polymerized with a monomer selected from the group consisting of acrylic acid, acrylamide, methacrylic acid, vinylacetic acid, 4-vinylbenzoic acid, itaconic acid, allyl amine, allylethylamine, 4-aminostyrene, 2-aminoethyl methacrylate, acryloyl chloride, methacryloyl chloride, chlorostyrene, dichlorostyrene, 4-hydroxystyrene, hydroxymethyl styrene, vinylbenzyl alcohol, allyl alcohol, 2-hydroxyethyl methacrylate, poly(ethylene glycol) methacrylate, and mixtures thereof, together with a monomer selected from the group consisting of acrylic acid, methacrylic acid, vinylacetic acid, 4-vinylbenzoic acid, itaconic acid, allyl amine, allylethylamine, 4-aminostyrene, 2-aminoethyl methacrylate, acryloyl chloride, methacryloyl chloride, chlorostyrene, dichlorostyrene, 4-hydroxystyrene, hydroxymethyl styrene, vinylbenzyl alcohol, allyl alcohol, 2-hydroxyethyl methacrylate, poly(ethylene glycol) methacrylate, methyl acrylate, methyl methacrylate, ethyl acrylate, ethyl methacrylate, styrene, 1-vinylimidazole, 2-vinylpyridine, 4-vinylpyridine, divinylbenzene, ethylene glycol dimethacrylate, N,N'-methylenediacrylamide, N,N'-phenylenediacrylamide, 3,5-bis(acryloylamido) benzoic acid, pentaerythritol triacrylate, trimethylolpropane trimethacrylate, pentaerytrithol tetraacrylate, trimethylolpropane ethoxylate (14/3 EO/OH) triacrylate, trimethylolpro-

pane ethoxylate (7/3 EO/OH) triacrylate, trimethylolpropane propoxylate (1 PO/OH) triacrylate, trimethylolpropane propoxylate (2 PO/OH) triacrylate, and mixtures thereof.

17. The device according to claim **1**, wherein the linker or support is non-hydrolyzable.

18. The device according to claim **1**, wherein the array is reusable for repeatedly hybridizing oligonucleotides to the array of capture oligonucleotides probes on the solid support.

19. The device according to claim **1**, wherein each of the capture oligonucleotide probes on the solid support has a different nucleotide sequence.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 7,083,917 B2
APPLICATION NO. : 09/963920
DATED : August 1, 2006
INVENTOR(S) : Francis Barany et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 1 at lines 10-13, delete "This invention was developed with government funding under National Institutes of Health Grant Nos. GM-41337-06, GM-43552-05, GM-42722-07, and GM-51628-02. The U.S. Government may have certain rights." and insert --This invention was made with government support under grant GM-41337-06 awarded by National Institutes of Health. The government has certain rights in the invention-- in its place.

Signed and Sealed this

Twenty-sixth Day of January, 2010

David J. Kappos

David J. Kappos
Director of the United States Patent and Trademark Office

Exhibit C

US007166434B2

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

(10) **Patent No.: US 7,166,434 B2**
(45) **Date of Patent: *Jan. 23, 2007**

(54) **DETECTION OF NUCLEIC ACID SEQUENCE DIFFERENCES USING COUPLED LIGASE DETECTION AND POLYMERASE CHAIN REACTIONS**

(75) Inventors: **Francis Barany**, New York, NY (US); **Matthew Lubin**, Rye Brook, NY (US); **George Barany**, Falcon Heights, MN (US); **Robert P. Hammer**, Baton Rouge, LA (US)

(73) Assignees: **Cornell Research Foundation, Inc.**, Ithaca, NY (US); **Board of Supervisors of Louisiana State University and Agricultural and Mechanical College**, Baton Rouge, LA (US); **Regents of the University of Minnesota**, Minneapolis, MN (US)

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

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **10/843,720**

(22) Filed: **May 12, 2004**

(65) **Prior Publication Data**

US 2004/0203061 A1 Oct. 14, 2004

Related U.S. Application Data

(60) Continuation of application No. 09/918,156, filed on Jul. 30, 2001, now Pat. No. 6,797,470, which is a continuation of application No. 09/440,523, filed on Nov. 15, 1999, now Pat. No. 6,268,148, which is a division of application No. 08/864,473, filed on May 28, 1997, now Pat. No. 6,027,889.

(60) Provisional application No. 60/018,532, filed on May 29, 1996.

(51) **Int. Cl.**
C12Q 1/68 (2006.01)
C12P 19/34 (2006.01)

(52) **U.S. Cl.** **435/6**; 435/91.1; 435/91.2

(58) **Field of Classification Search** 435/6, 435/91.1, 91.2
See application file for complete search history.

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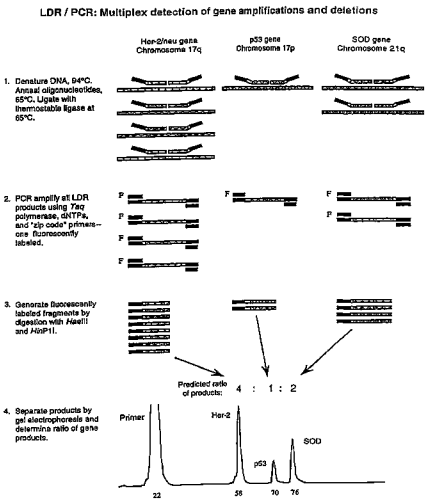
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Primary Examiner—Kenneth R. Horlick
(74) *Attorney, Agent, or Firm*—Nixon Peabody LLP

(57) **ABSTRACT**

The present invention relates to the detection of nucleic acid sequence differences using coupled ligase detection reaction and polymerase chain reaction. One aspect of the present invention involves use of a ligase detection reaction coupled to a polymerase chain reaction. Another aspect of the present invention relates to the use of a primary polymerase chain reaction coupled to a secondary polymerase chain reaction coupled to a ligase detection reaction. A third aspect of the present invention involves a primary polymerase chain reaction coupled to a secondary polymerase chain reaction. Such coupling of the ligase detection reaction and the polymerase chain reaction permits multiplex detection of nucleic acid sequence differences.

22 Claims, 29 Drawing Sheets



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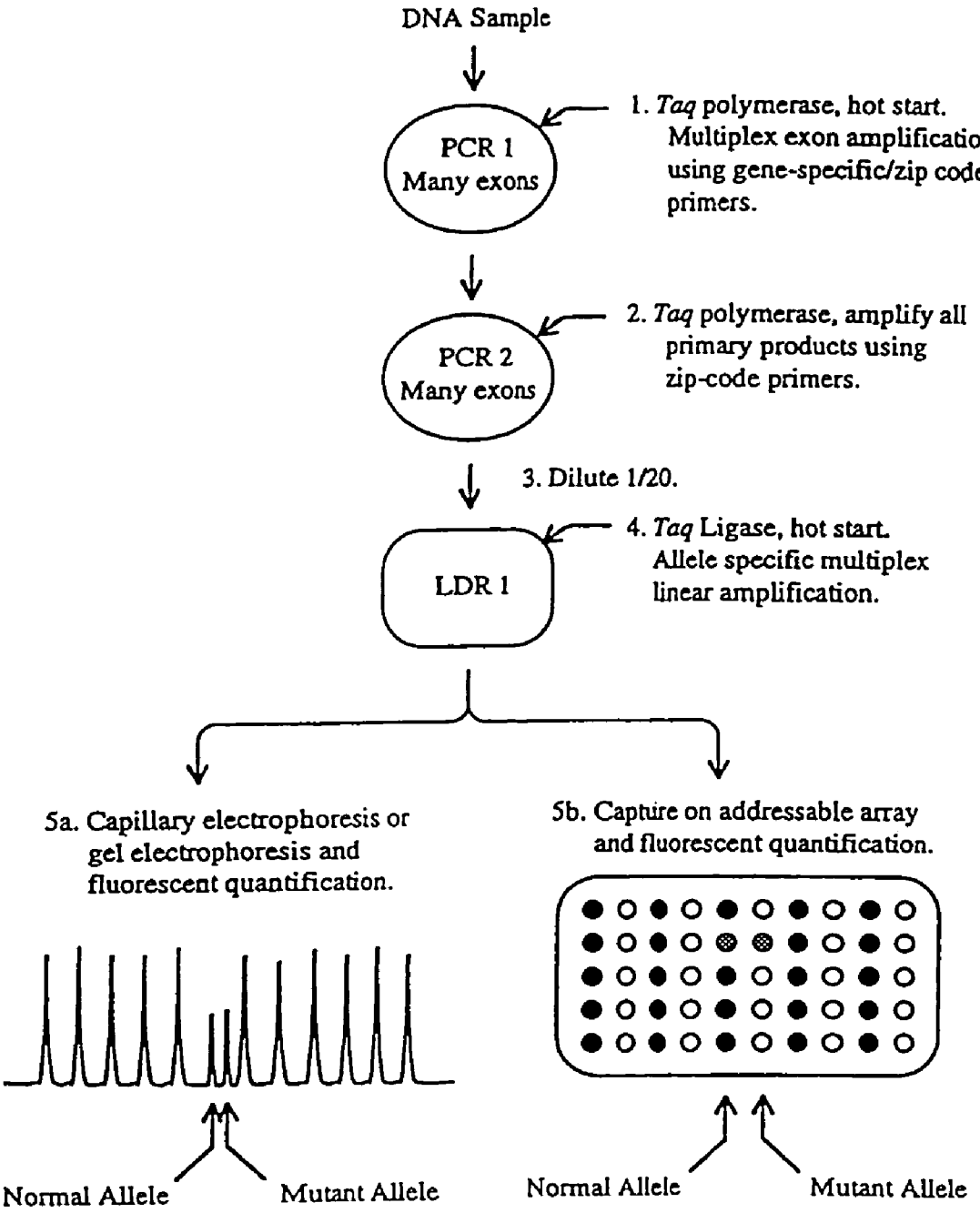

DNA Sample
PCR 1
Many exons
1. *Taq* polymerase, hot start. Multiplex exon amplification using gene-specific/zip code primers.
PCR 2
Many exons
2. *Taq* polymerase, amplify all primary products using zip-code primers.
3. Dilute 1/20.
LDR 1
4. *Taq* Ligase, hot start. Allele specific multiplex linear amplification.
5a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
5b. Capture on addressable array and fluorescent quantification.
Normal Allele
Mutant Allele
Normal Allele
Mutant Allele

FIG. 1

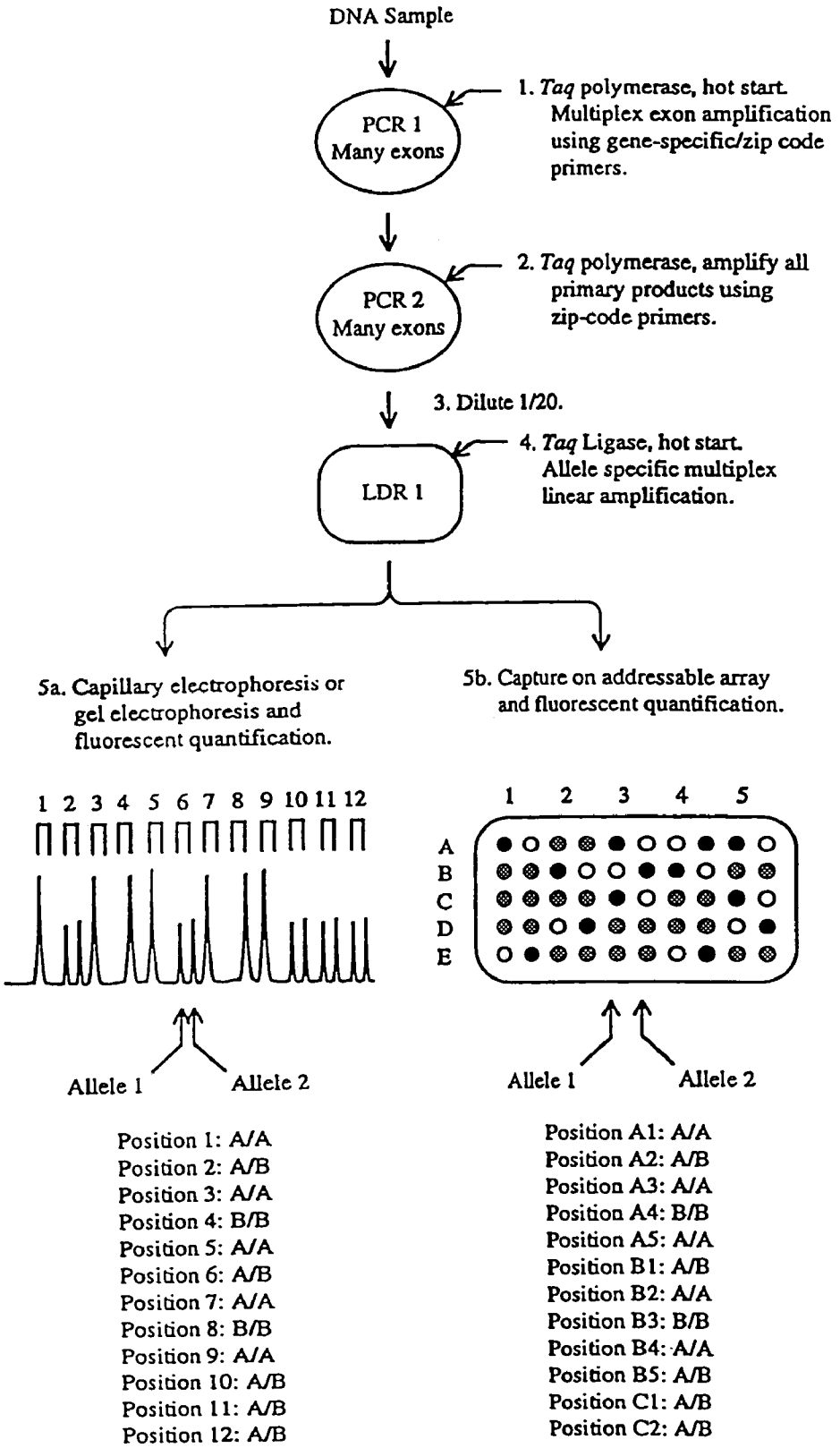

DNA Sample
PCR 1
Many exons
1. Taq polymerase, hot start. Multiplex exon amplification using gene-specific/zip code primers.
PCR 2
Many exons
2. Taq polymerase, amplify all primary products using zip-code primers.
3. Dilute 1/20.
LDR 1
4. Taq Ligase, hot start. Allele specific multiplex linear amplification.
5a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
5b. Capture on addressable array and fluorescent quantification.
1 2 3 4 5 6 7 8 9 10 11 12
Allele 1
Allele 2
1 2 3 4 5
A
B
C
D
E
Allele 1
Allele 2
Position 1: A/A
Position 2: A/B
Position 3: A/A
Position 4: B/B
Position 5: A/A
Position 6: A/B
Position 7: A/A
Position 8: B/B
Position 9: A/A
Position 10: A/B
Position 11: A/B
Position 12: A/B
Position A1: A/A
Position A2: A/B
Position A3: A/A
Position A4: B/B
Position A5: A/A
Position B1: A/B
Position B2: A/A
Position B3: B/B
Position B4: A/A
Position B5: A/B
Position C1: A/B
Position C2: A/B

FIG. 2

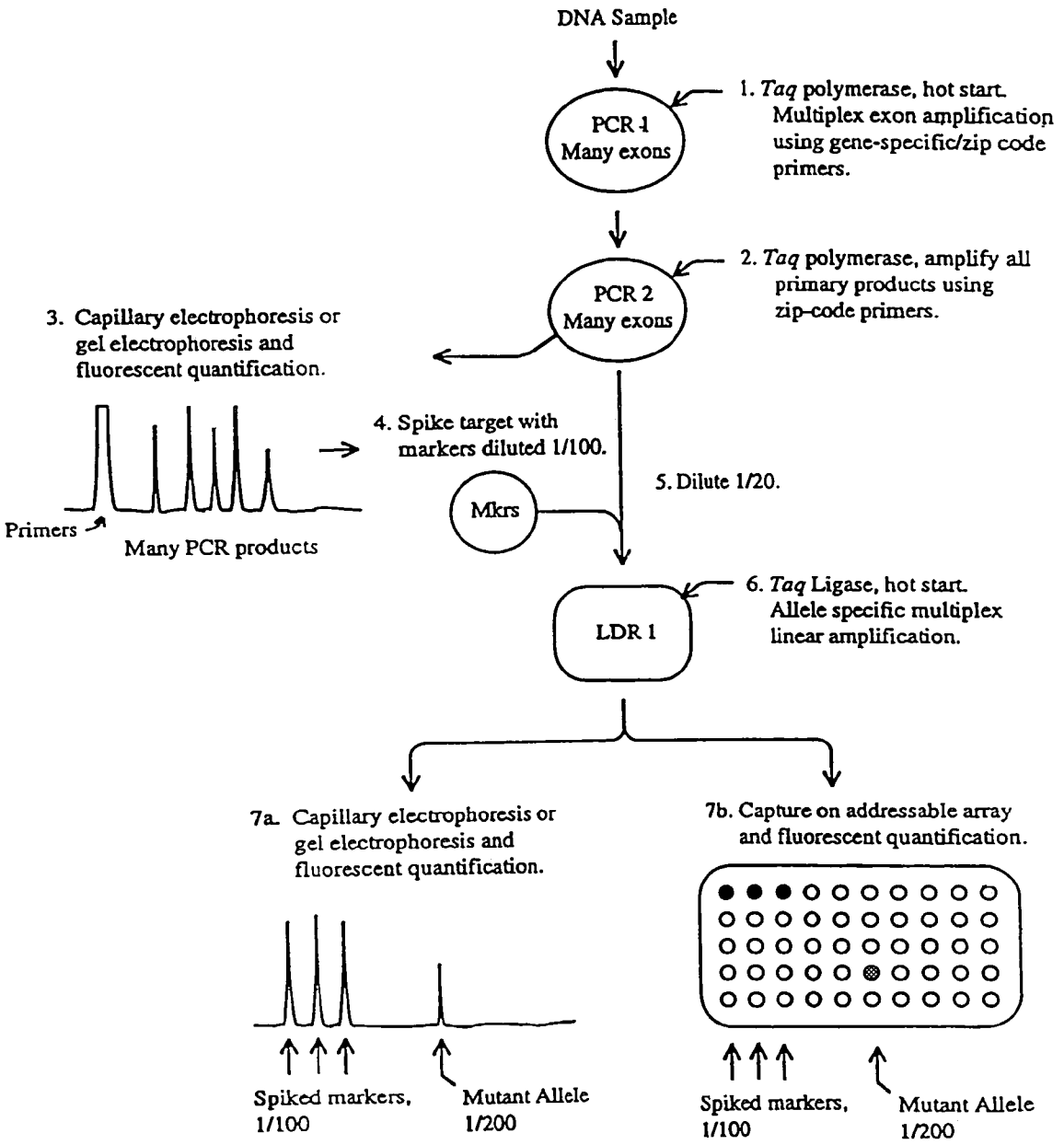

DNA Sample
PCR 1
Many exons
1. Taq polymerase, hot start. Multiplex exon amplification using gene-specific/zip code primers.
PCR 2
Many exons
2. Taq polymerase, amplify all primary products using zip-code primers.
3. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Primers
Many PCR products
4. Spike target with markers diluted 1/100.
Mkrs
5. Dilute 1/20.
LDR 1
6. Taq Ligase, hot start. Allele specific multiplex linear amplification.
7a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200
7b. Capture on addressable array and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200

FIG. 3

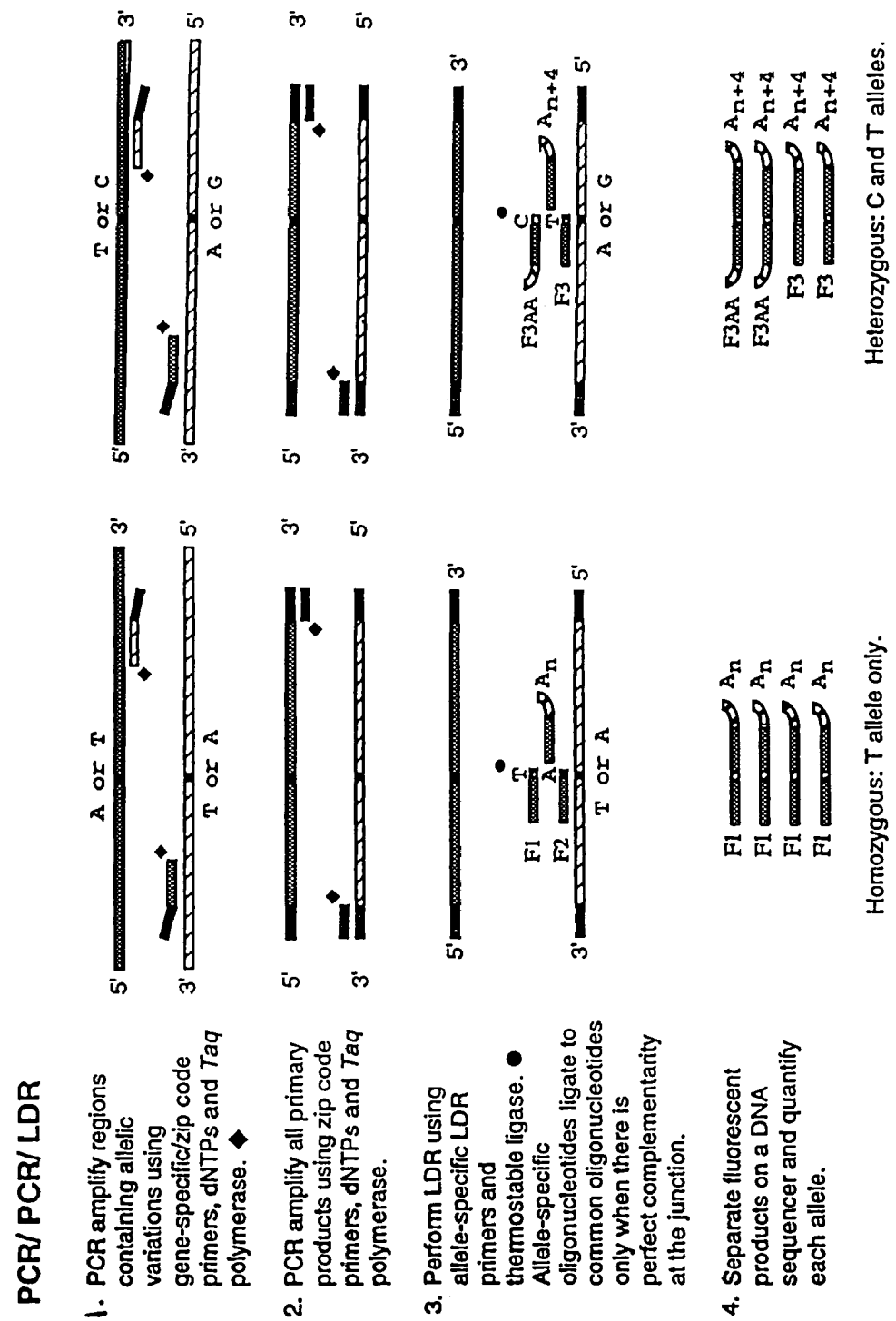

PCR/ PCR/ LDR
1. PCR amplify regions containing allelic variations using gene-specific/zip code primers, dNTPs and Taq polymerase. ◆
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase.
3. Perform LDR using allele-specific LDR primers and thermostable ligase. ● Allele-specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
4. Separate fluorescent products on a DNA sequencer and quantify each allele.
A or T
T or A
T or C
A or G
5'
3'
F1
F2
F3
F3AA
An
An+4
Homozygous: T allele only.
Heterozygous: C and T alleles.

FIG. 4

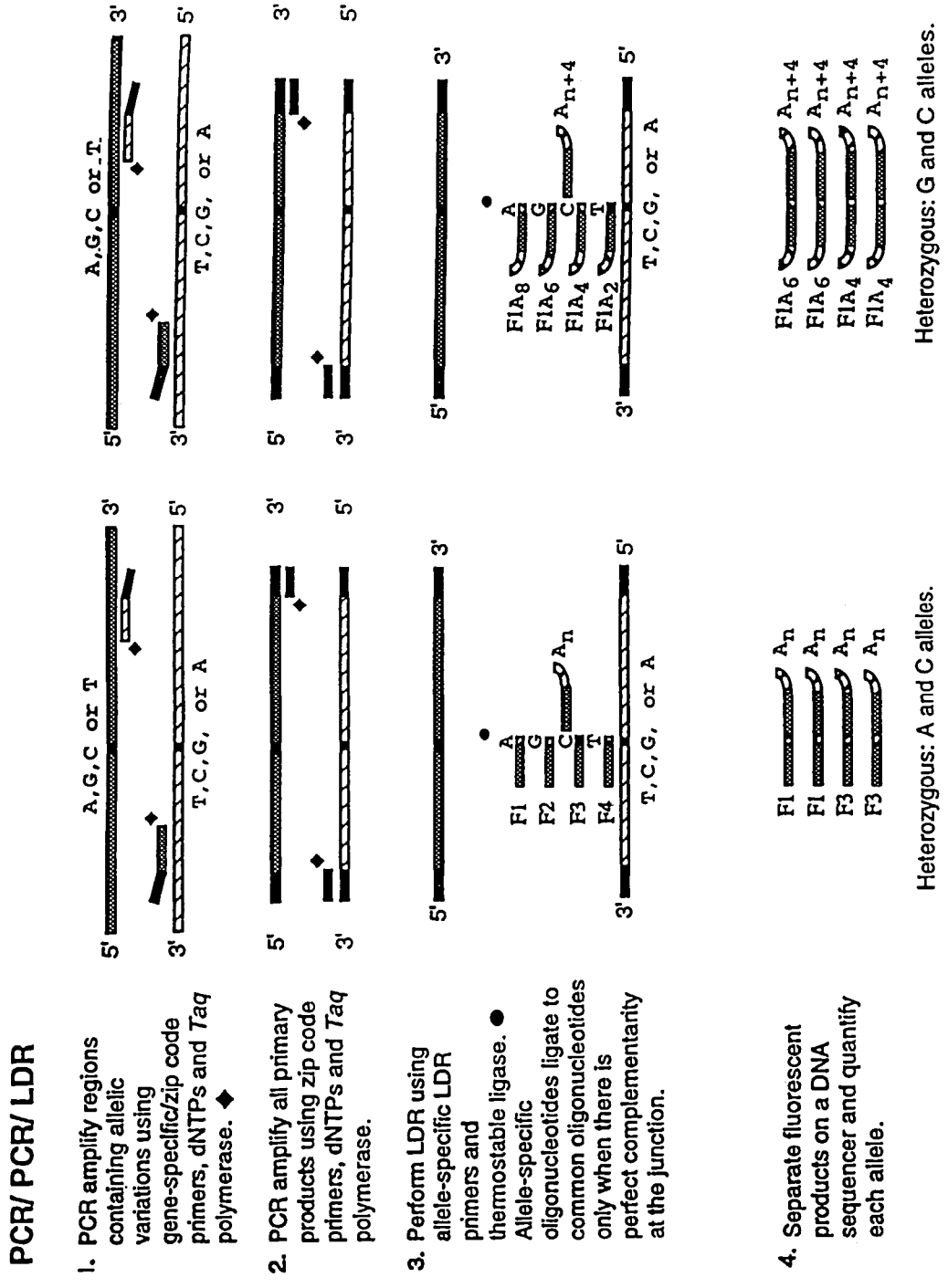

PCR/ PCR/ LDR
1. PCR amplify regions containing allelic variations using gene-specific/zip code primers, dNTPs and Taq polymerase. ◆
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase.
3. Perform LDR using allele-specific LDR primers and thermostable ligase. ● Allele-specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
4. Separate fluorescent products on a DNA sequencer and quantify each allele.
A,G,C or T
T,C,G, or A
F1
F2
F3
F4
Heterozygous: A and C alleles.
Heterozygous: G and C alleles.

FIG. 5

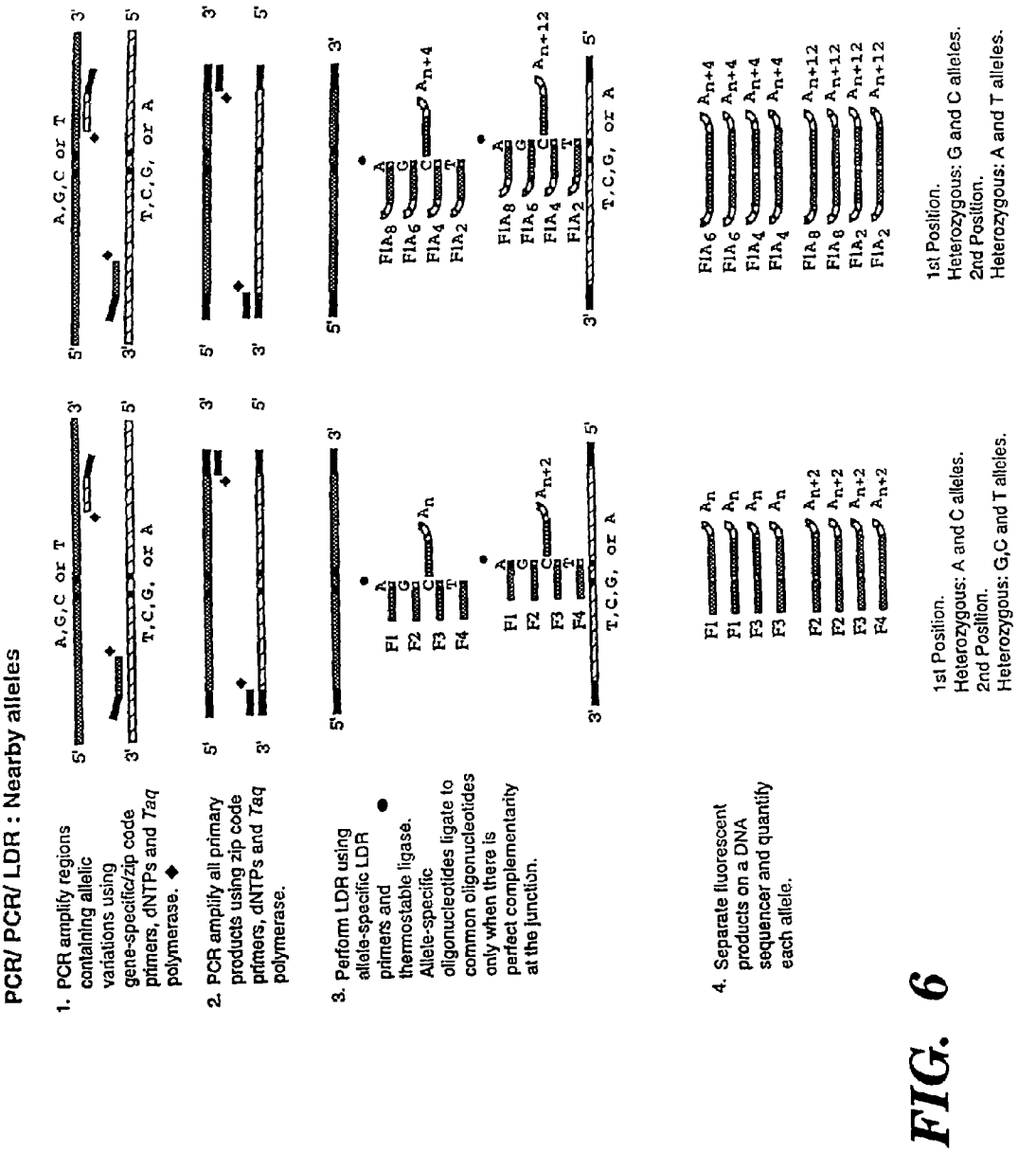

PCR/ PCR/ LDR : Nearby alleles
1. PCR amplify regions containing allelic variations using gene-specific/zip code primers, dNTPs and Taq polymerase. ◆
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase.
3. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele-specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction. ●
4. Separate fluorescent products on a DNA sequencer and quantify each allele.
A,G,C or T
T,C,G, or A
F1
F2
F3
F4
1st Position.
Heterozygous: A and C alleles.
2nd Position.
Heterozygous: G,C and T alleles.
1st Position.
Heterozygous: G and C alleles.
2nd Position.
Heterozygous: A and T alleles.

FIG. 6

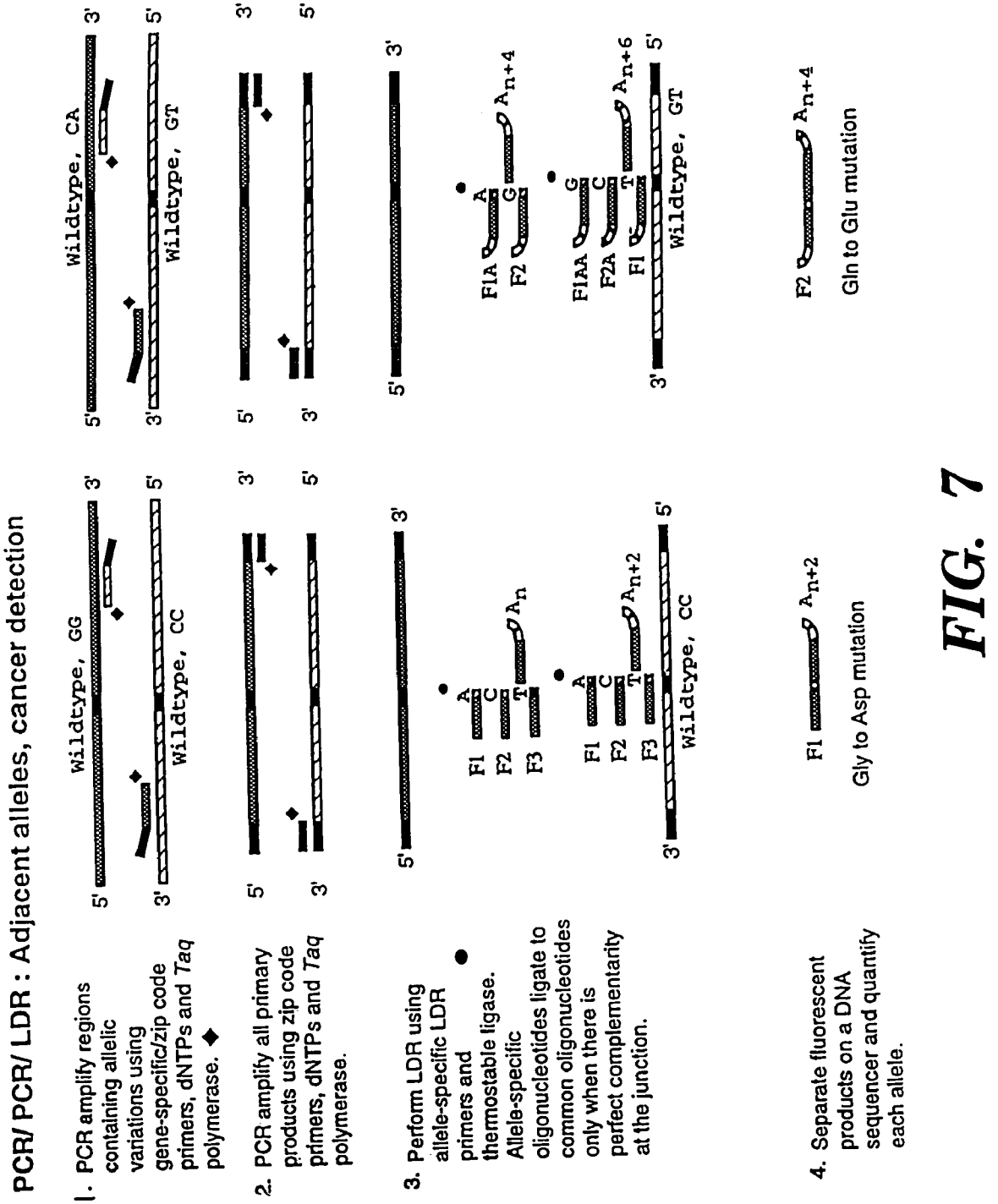

PCR/ PCR/ LDR : Adjacent alleles, cancer detection
1. PCR amplify regions containing allelic variations using gene-specific/zip code primers, dNTPs and Taq polymerase. ◆
Wildtype, GG
Wildtype, CC
Wildtype, CA
Wildtype, GT
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase.
3. Perform LDR using allele-specific LDR primers and ● thermostable ligase. Allele-specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
F1
F2
F3
F1A
F2A
F1AA
4. Separate fluorescent products on a DNA sequencer and quantify each allele.
Gly to Asp mutation
Gln to Glu mutation

FIG. 7

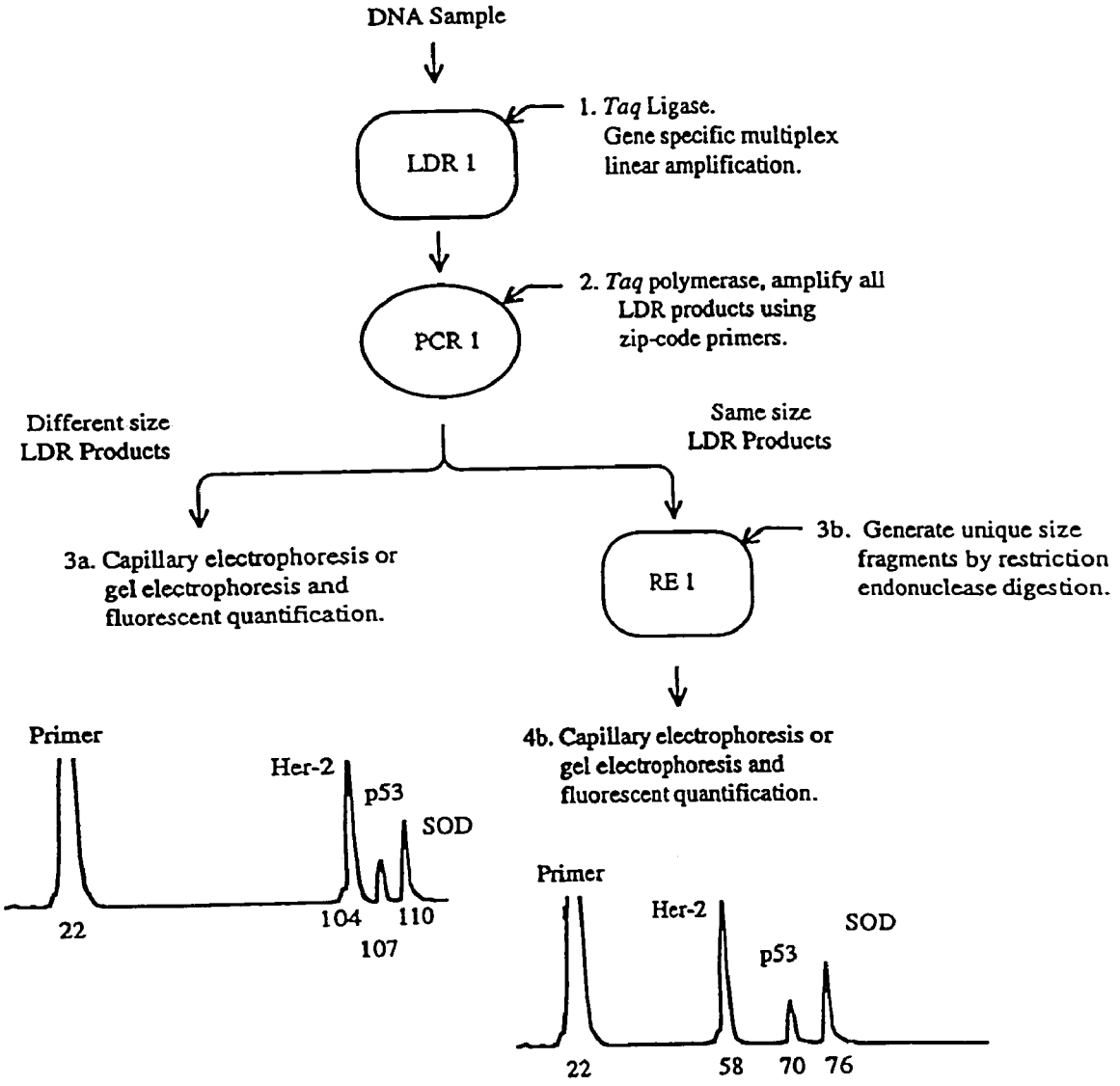

DNA Sample
LDR 1
1. Taq Ligase. Gene specific multiplex linear amplification.
PCR 1
2. Taq polymerase, amplify all LDR products using zip-code primers.
Different size LDR Products
Same size LDR Products
3a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
RE 1
3b. Generate unique size fragments by restriction endonuclease digestion.
4b. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Primer
Her-2
p53
SOD
22
104
107
110
Primer
Her-2
p53
SOD
22
58
70
76

FIG. 8

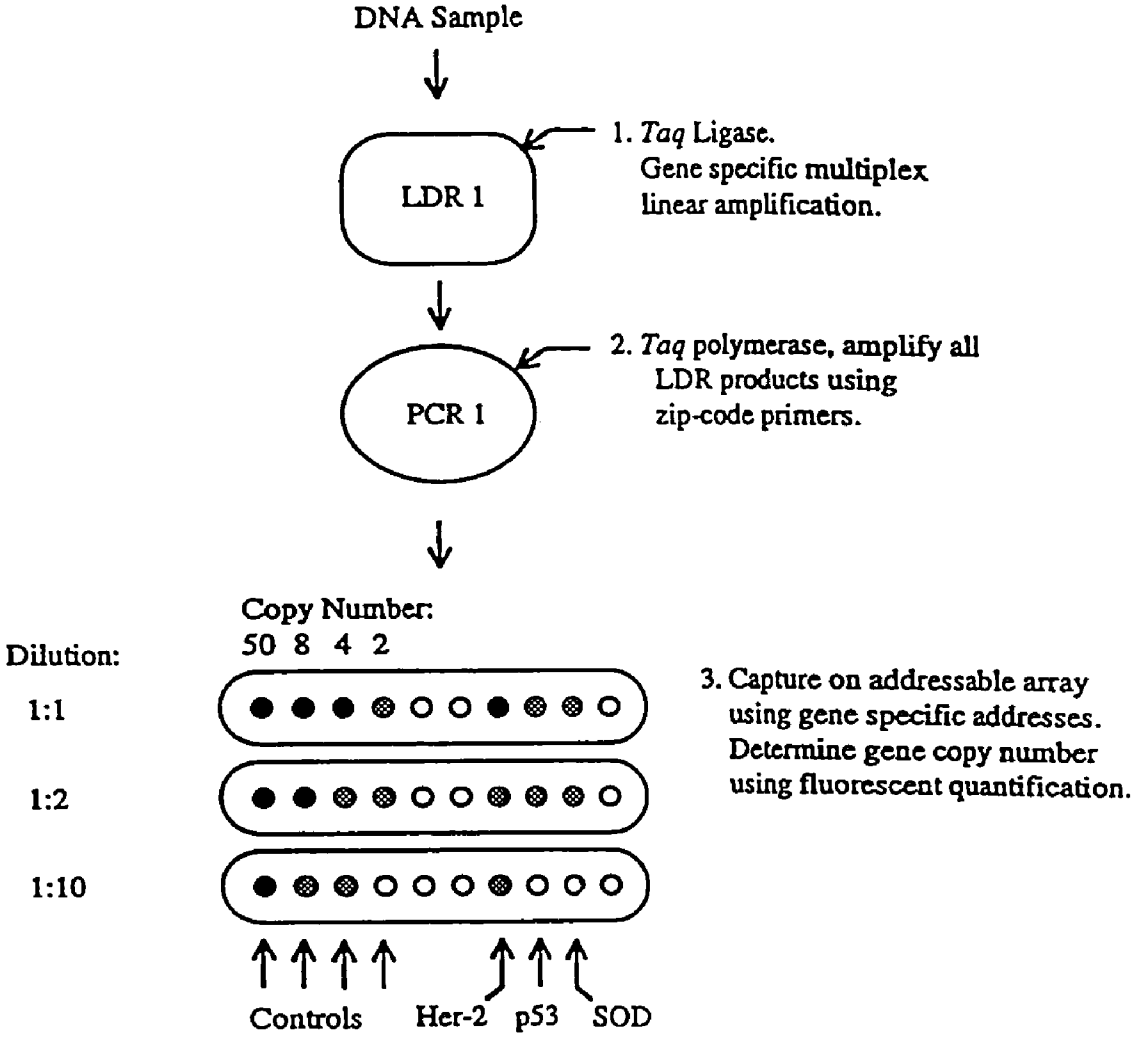

DNA Sample
LDR 1
1. Taq Ligase. Gene specific multiplex linear amplification.
PCR 1
2. Taq polymerase, amplify all LDR products using zip-code primers.
Copy Number:
50 8 4 2
Dilution:
1:1
1:2
1:10
3. Capture on addressable array using gene specific addresses. Determine gene copy number using fluorescent quantification.
Controls
Her-2
p53
SOD

FIG. 9

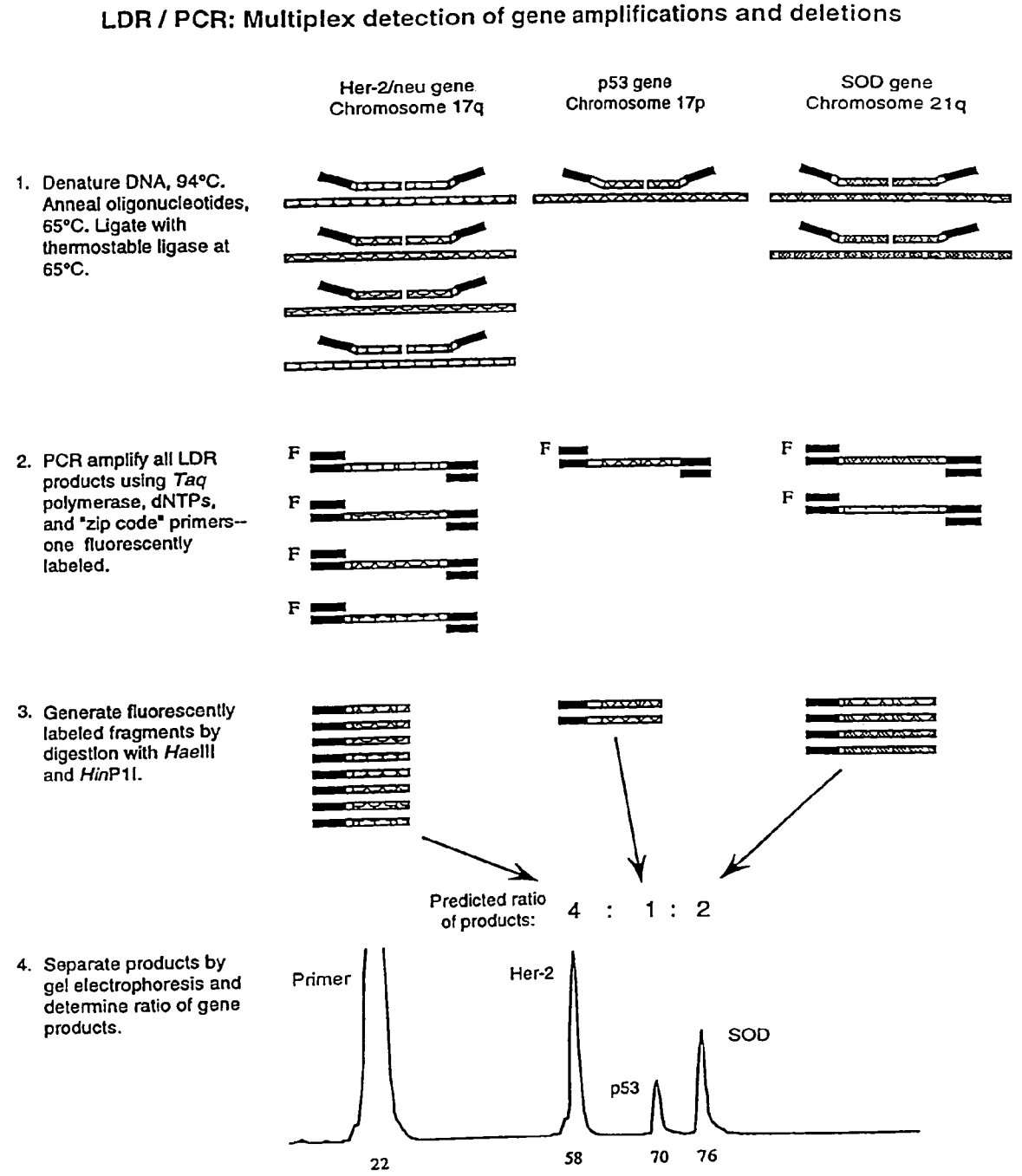

LDR / PCR: Multiplex detection of gene amplifications and deletions
Her-2/neu gene Chromosome 17q
p53 gene Chromosome 17p
SOD gene Chromosome 21q
1. Denature DNA, 94°C. Anneal oligonucleotides, 65°C. Ligate with thermostable ligase at 65°C.
2. PCR amplify all LDR products using Taq polymerase, dNTPs, and "zip code" primers--one fluorescently labeled.
F
3. Generate fluorescently labeled fragments by digestion with HaeIII and HinP1I.
Predicted ratio of products: 4 : 1 : 2
4. Separate products by gel electrophoresis and determine ratio of gene products.
Primer
Her-2
p53
SOD
22
58
70
76

FIG. 10

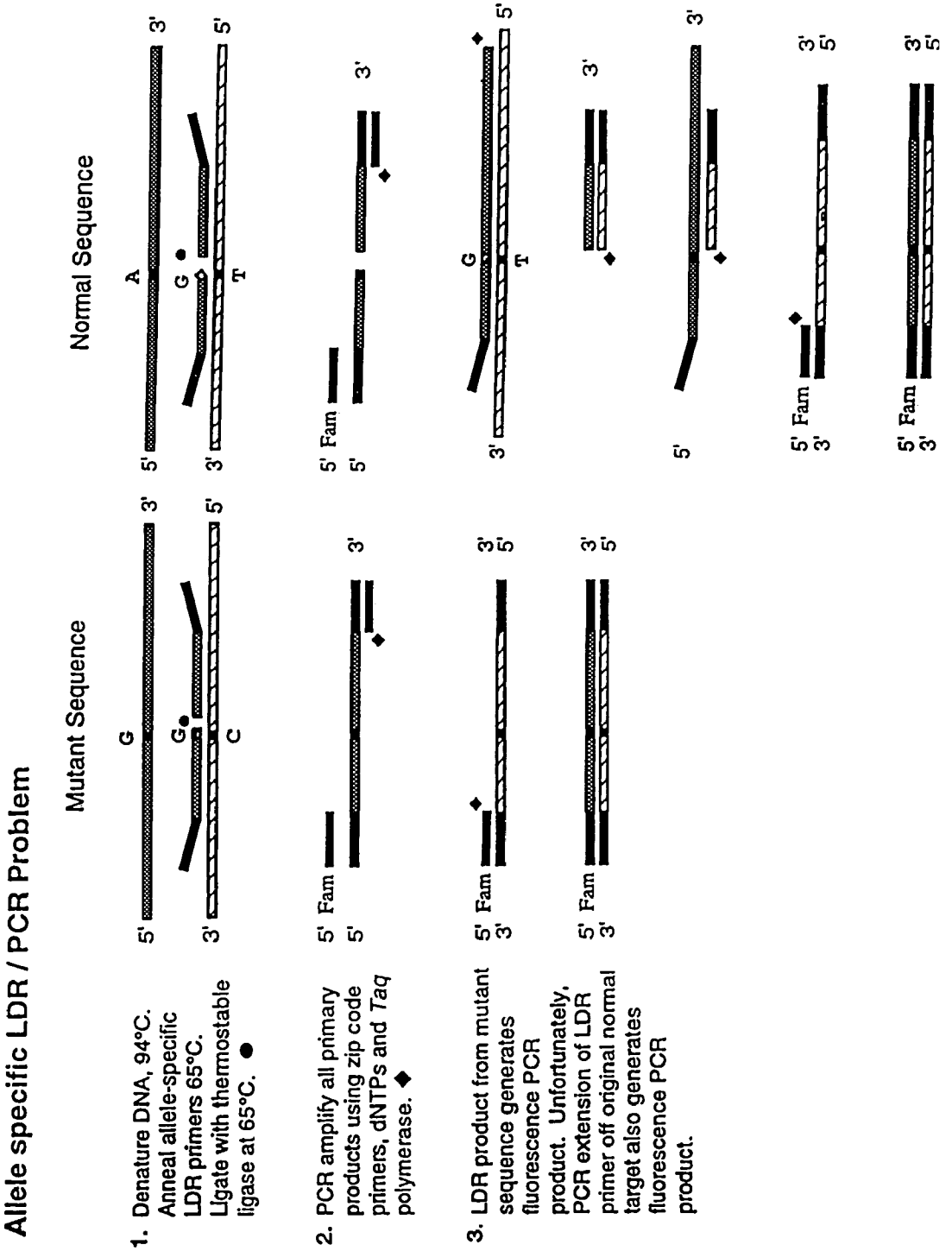

Allele specific LDR / PCR Problem
Mutant Sequence
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific LDR primers 65°C. Ligate with thermostable ligase at 65°C. ●
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
3. LDR product from mutant sequence generates fluorescence PCR product. Unfortunately, PCR extension of LDR primer off original normal target also generates fluorescence PCR product.
5' Fam
3' 5'

FIG. 11

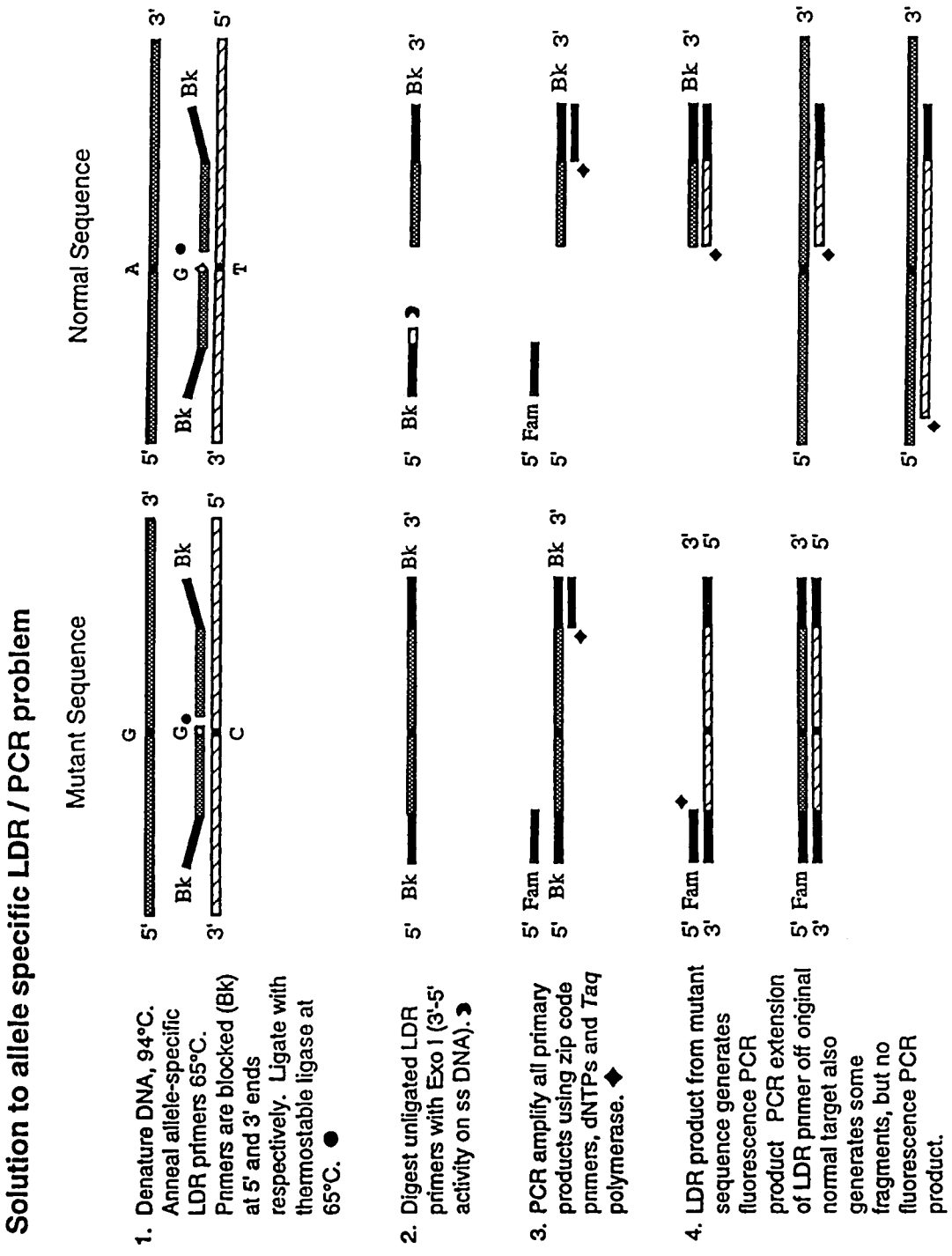

Solution to allele specific LDR / PCR problem
Mutant Sequence
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific LDR primers 65°C. Primers are blocked (Bk) at 5' and 3' ends respectively. Ligate with thermostable ligase at 65°C. ●
2. Digest unligated LDR primers with Exo I (3'-5' activity on ss DNA). ◗
3. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
4. LDR product from mutant sequence generates fluorescence PCR product PCR extension of LDR primer off original normal target also generates some fragments, but no fluorescence PCR product.
5' 3' G A T C Bk Fam

FIG. 12

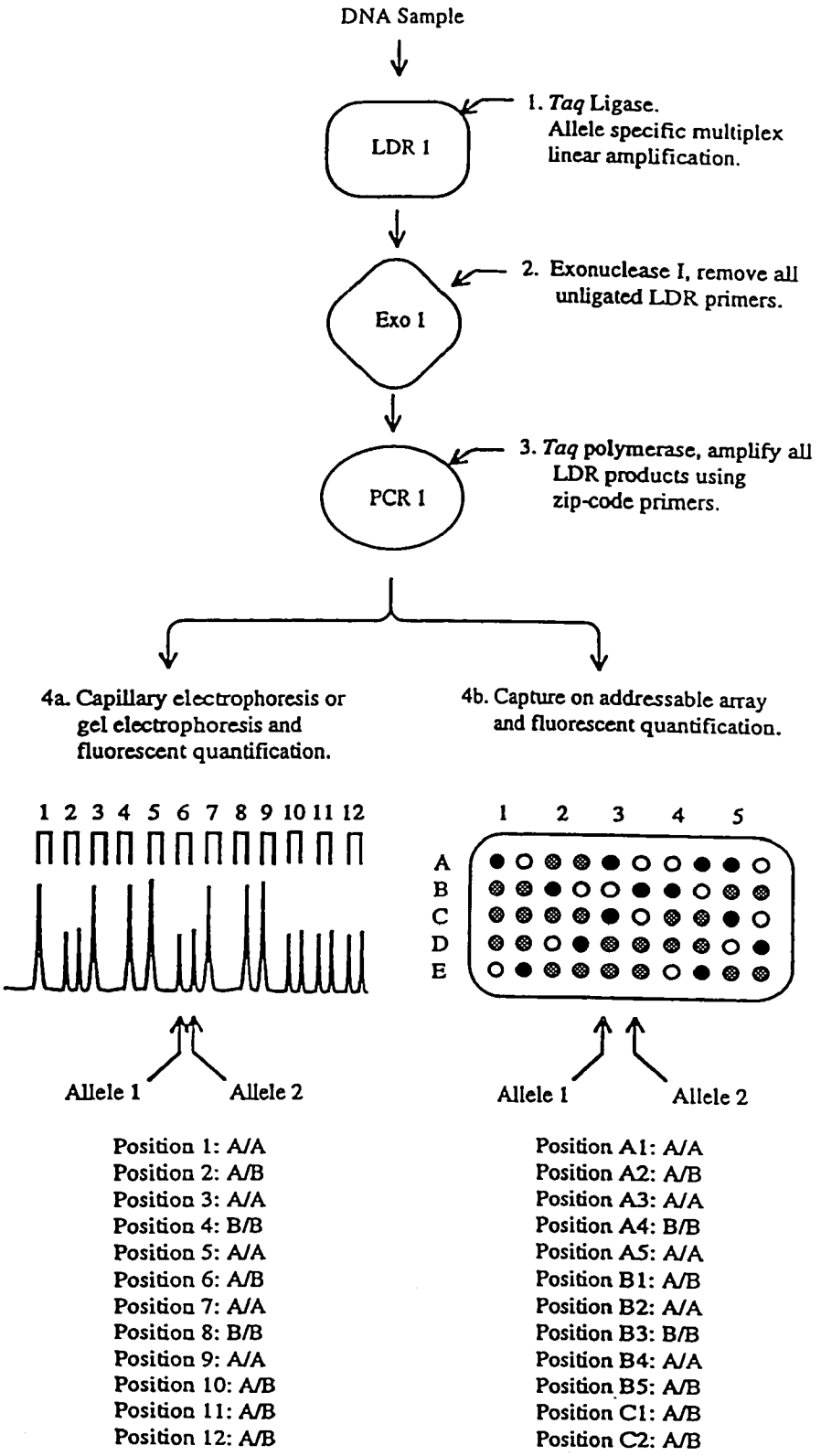

DNA Sample
LDR 1
1. Taq Ligase. Allele specific multiplex linear amplification.
Exo 1
2. Exonuclease I, remove all unligated LDR primers.
PCR 1
3. Taq polymerase, amplify all LDR products using zip-code primers.
4a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
4b. Capture on addressable array and fluorescent quantification.
1 2 3 4 5 6 7 8 9 10 11 12
Allele 1
Allele 2
Position 1: A/A
Position 2: A/B
Position 3: A/A
Position 4: B/B
Position 5: A/A
Position 6: A/B
Position 7: A/A
Position 8: B/B
Position 9: A/A
Position 10: A/B
Position 11: A/B
Position 12: A/B
1 2 3 4 5
A
B
C
D
E
Allele 1
Allele 2
Position A1: A/A
Position A2: A/B
Position A3: A/A
Position A4: B/B
Position A5: A/A
Position B1: A/B
Position B2: A/A
Position B3: B/B
Position B4: A/A
Position B5: A/B
Position C1: A/B
Position C2: A/B

FIG. 13

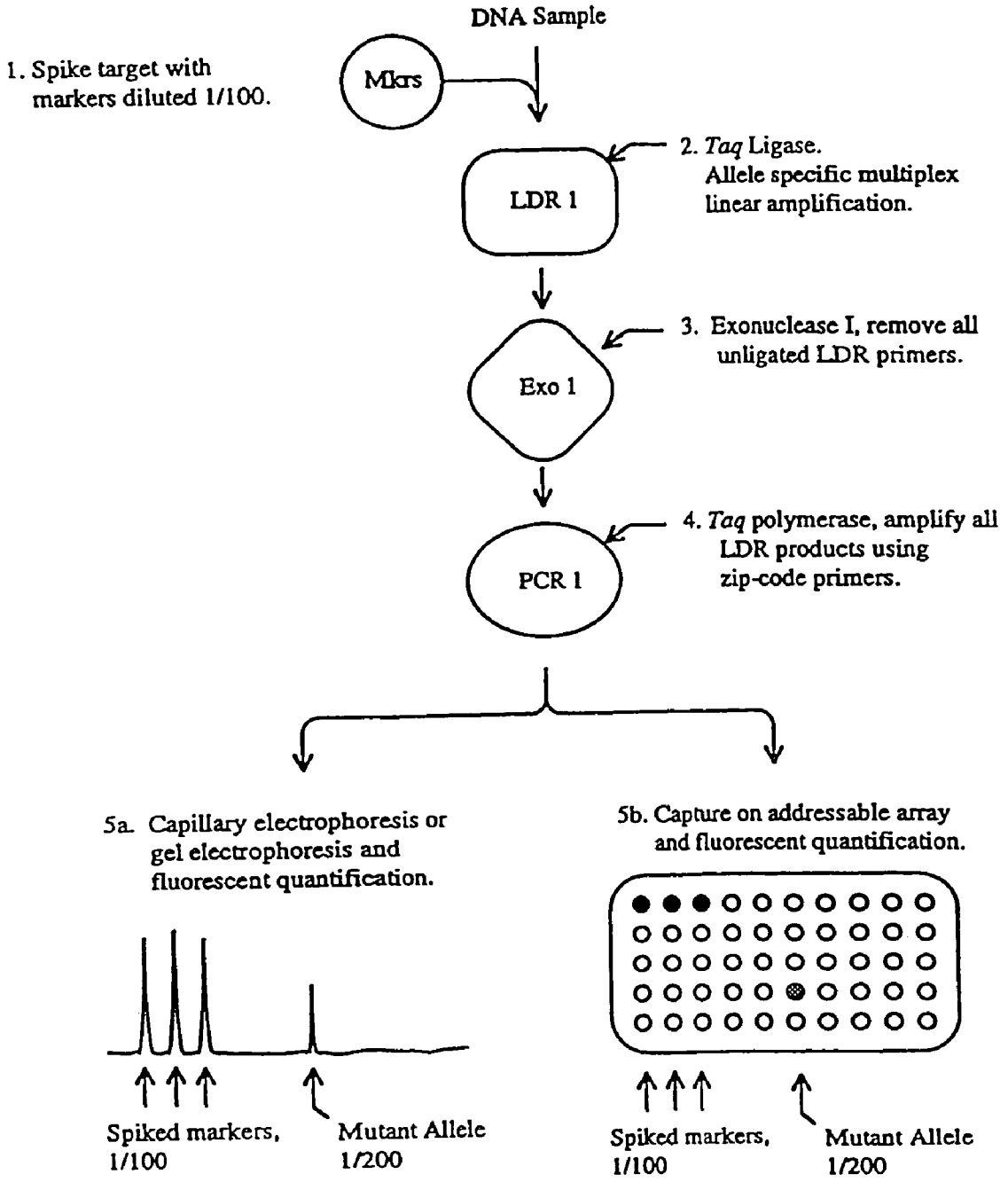

DNA Sample
1. Spike target with markers diluted 1/100.
Mkrs
LDR 1
2. Taq Ligase. Allele specific multiplex linear amplification.
Exo 1
3. Exonuclease I, remove all unligated LDR primers.
PCR 1
4. Taq polymerase, amplify all LDR products using zip-code primers.
5a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200
5b. Capture on addressable array and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200

FIG. 14

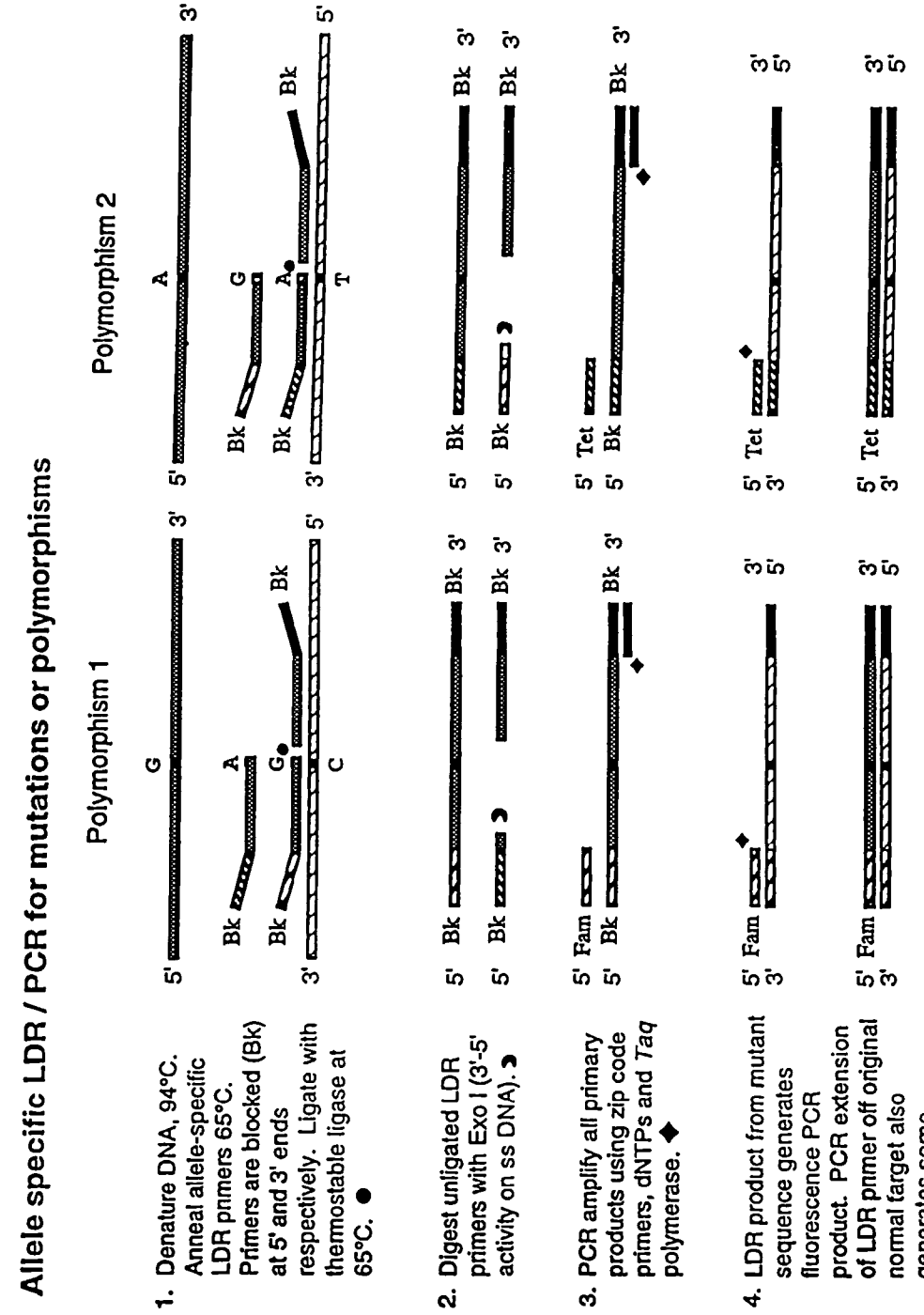

Allele specific LDR / PCR for mutations or polymorphisms
Polymorphism 1
Polymorphism 2
1. Denature DNA, 94°C. Anneal allele-specific LDR primers 65°C. Primers are blocked (Bk) at 5' and 3' ends respectively. Ligate with thermostable ligase at 65°C. ●
2. Digest unligated LDR primers with Exo I (3'-5' activity on ss DNA). ◗
3. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
4. LDR product from mutant sequence generates fluorescence PCR product. PCR extension of LDR primer off original normal target also generates some fragments, but no fluorescence PCR product.
5'
3'
Bk
Fam
Tet
G
A
C
T

FIG. 15

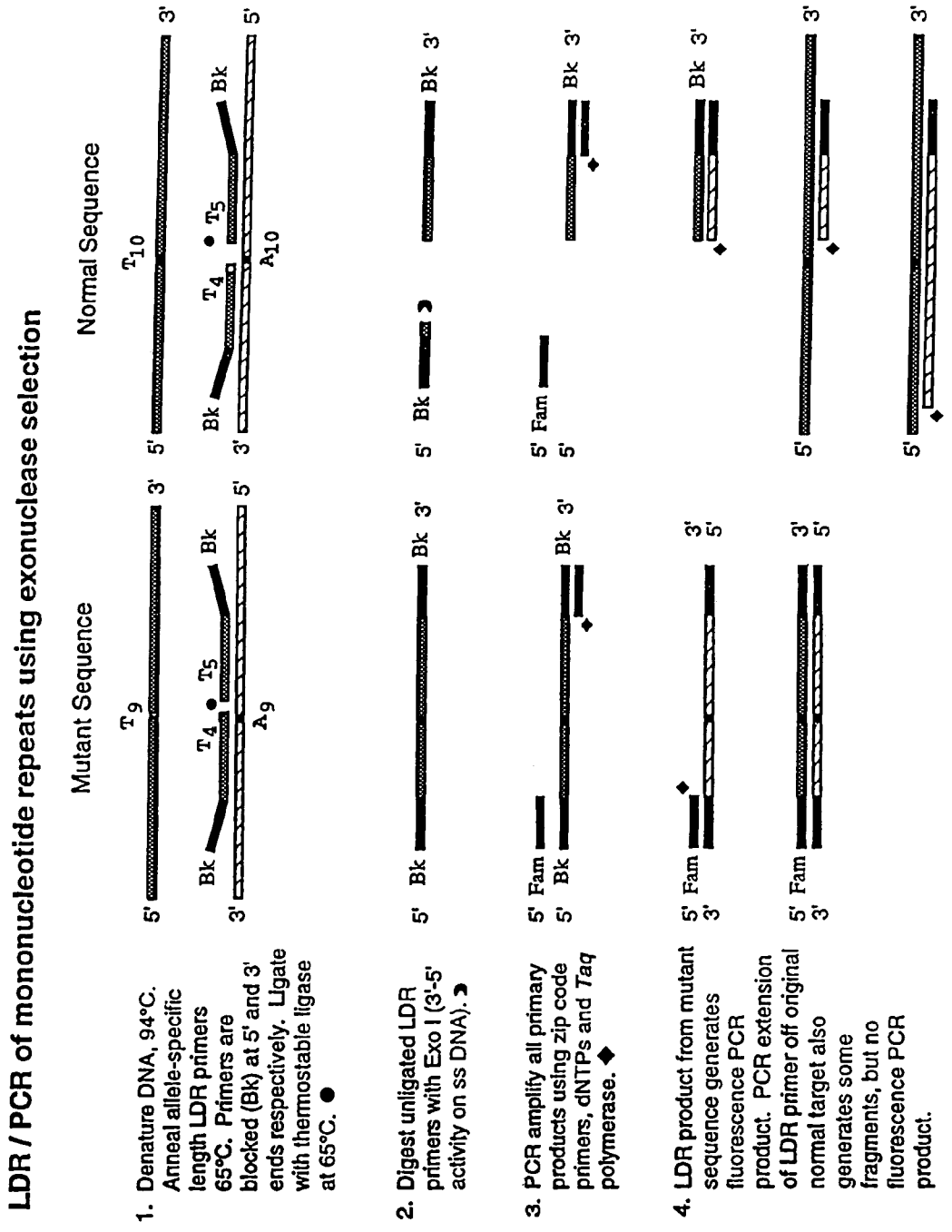

LDR / PCR of mononucleotide repeats using exonuclease selection
Mutant Sequence
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific length LDR primers 65°C. Primers are blocked (Bk) at 5' and 3' ends respectively. Ligate with thermostable ligase at 65°C. ●
2. Digest unligated LDR primers with Exo I (3'-5' activity on ss DNA). ➲
3. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
4. LDR product from mutant sequence generates fluorescence PCR product. PCR extension of LDR primer off original normal target also generates some fragments, but no fluorescence PCR product.
T9
T10
T4
T5
A9
A10
Bk
Fam
5'
3'

FIG. 16

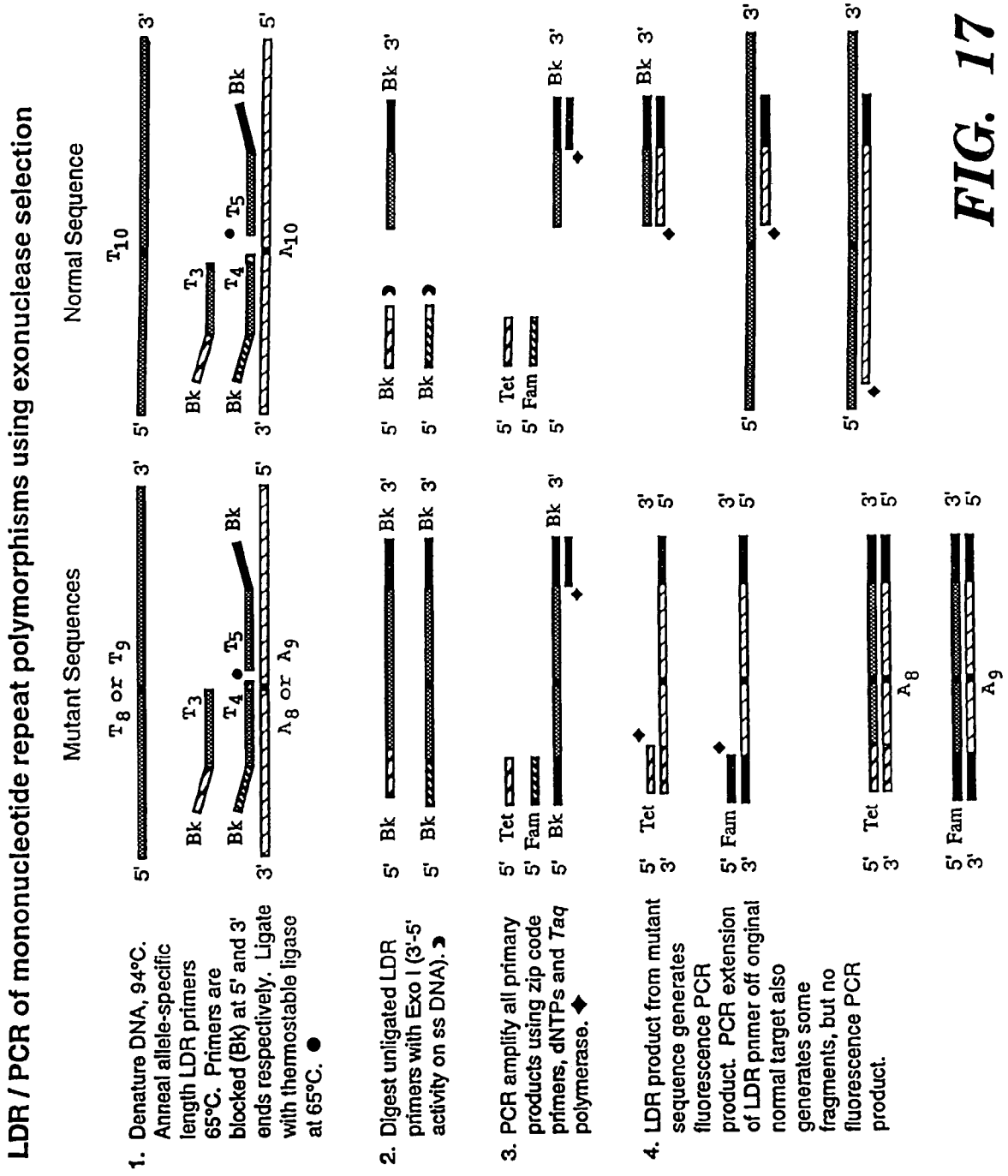

LDR / PCR of mononucleotide repeat polymorphisms using exonuclease selection
Mutant Sequences
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific length LDR primers 65°C. Primers are blocked (Bk) at 5' and 3' ends respectively. Ligate with thermostable ligase at 65°C. ●
2. Digest unligated LDR primers with Exo I (3'-5' activity on ss DNA). ❯
3. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
4. LDR product from mutant sequence generates fluorescence PCR product. PCR extension of LDR pnmer off onginal normal target also generates some fragments, but no fluorescence PCR product.
T8 or T9
A8 or A9
T10
A10
T3
T4
T5
Bk
Tet
Fam

FIG. 17

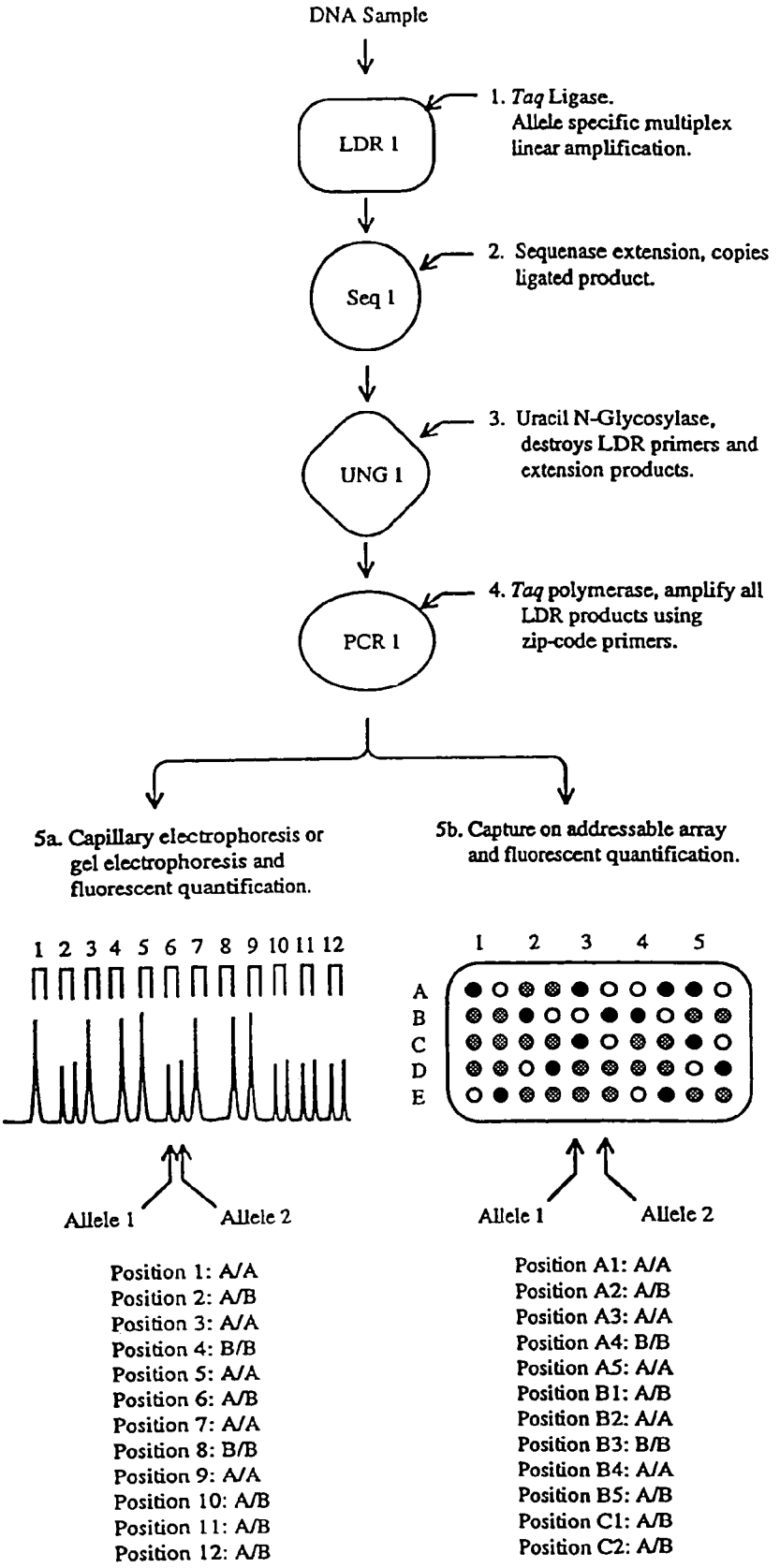

DNA Sample
LDR 1
1. Taq Ligase. Allele specific multiplex linear amplification.
Seq 1
2. Sequenase extension, copies ligated product.
UNG 1
3. Uracil N-Glycosylase, destroys LDR primers and extension products.
PCR 1
4. Taq polymerase, amplify all LDR products using zip-code primers.
5a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
5b. Capture on addressable array and fluorescent quantification.
1 2 3 4 5 6 7 8 9 10 11 12
Allele 1
Allele 2
Position 1: A/A
Position 2: A/B
Position 3: A/A
Position 4: B/B
Position 5: A/A
Position 6: A/B
Position 7: A/A
Position 8: B/B
Position 9: A/A
Position 10: A/B
Position 11: A/B
Position 12: A/B
1 2 3 4 5
A
B
C
D
E
Allele 1
Allele 2
Position A1: A/A
Position A2: A/B
Position A3: A/A
Position A4: B/B
Position A5: A/A
Position B1: A/B
Position B2: A/A
Position B3: B/B
Position B4: A/A
Position B5: A/B
Position C1: A/B
Position C2: A/B

FIG. 18

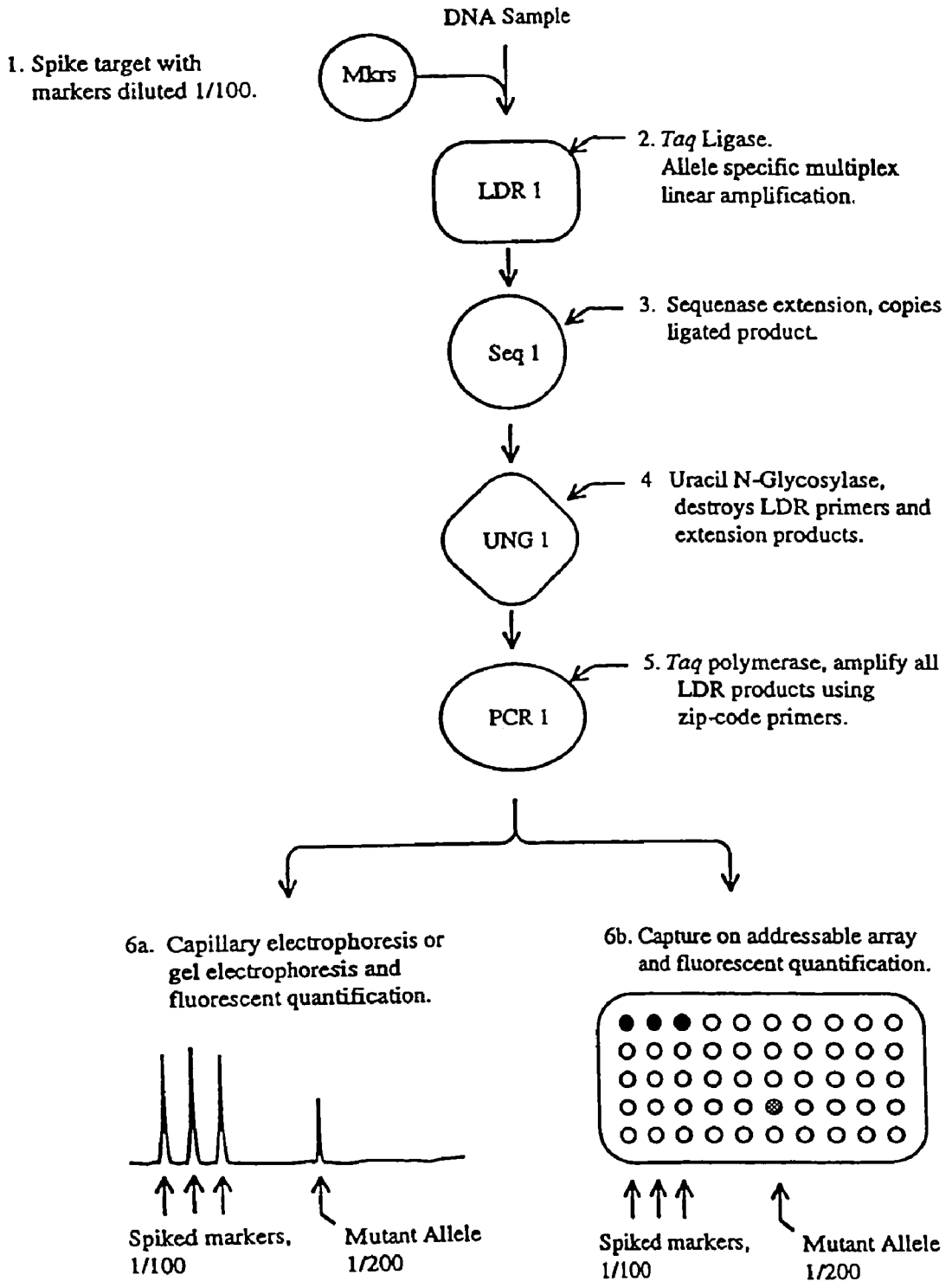

DNA Sample
1. Spike target with markers diluted 1/100.
Mkrs
LDR 1
2. Taq Ligase. Allele specific multiplex linear amplification.
Seq 1
3. Sequenase extension, copies ligated product.
UNG 1
4 Uracil N-Glycosylase, destroys LDR primers and extension products.
PCR 1
5. Taq polymerase, amplify all LDR products using zip-code primers.
6a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200
6b. Capture on addressable array and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200

FIG. 19

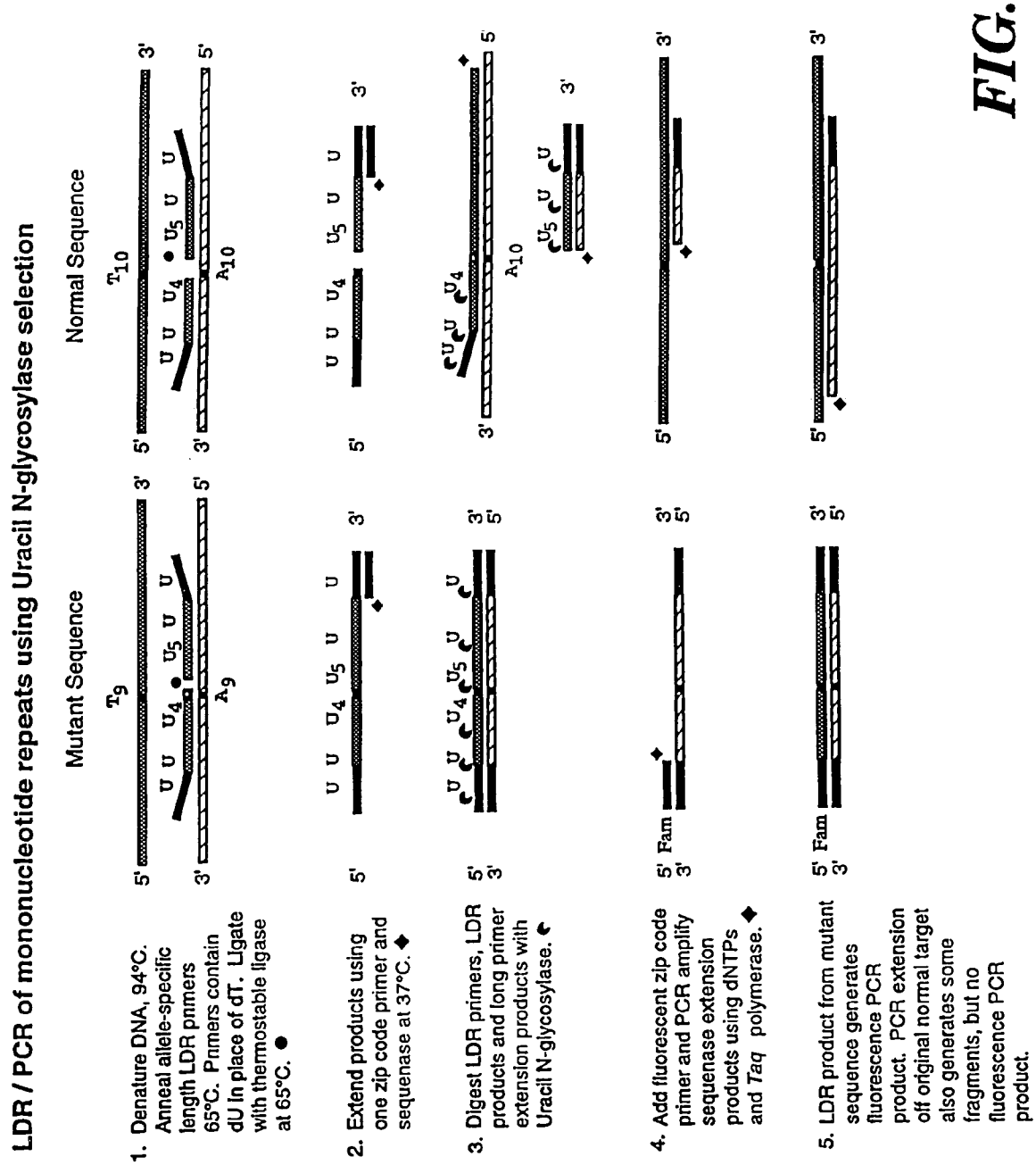

LDR / PCR of mononucleotide repeats using Uracil N-glycosylase selection
Mutant Sequence
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific length LDR pnmers 65°C. Pnmers contain dU In place of dT. Ligate with thermostable ligase at 65°C. ●
2. Extend products using one zip code primer and sequenase at 37°C. ◆
3. Digest LDR primers, LDR products and long primer extension products with Uracil N-glycosylase. ◖
4. Add fluorescent zip code primer and PCR amplify sequenase extension products using dNTPs ◆ and Taq polymerase.
5. LDR product from mutant sequence generates fluorescence PCR product. PCR extension off original normal target also generates some fragments, but no fluorescence PCR product.
Fam

FIG. 20

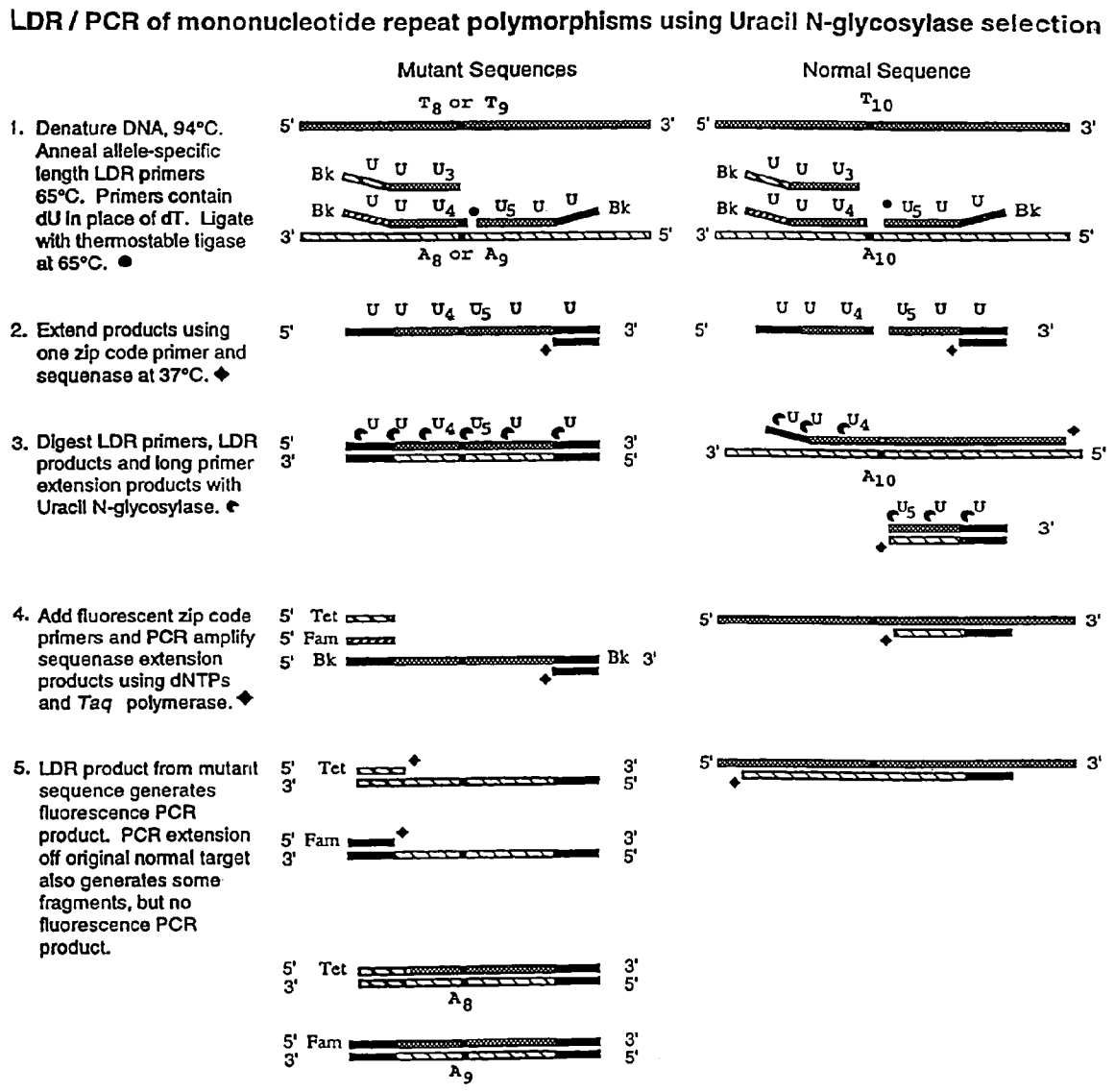

LDR / PCR of mononucleotide repeat polymorphisms using Uracil N-glycosylase selection
Mutant Sequences
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific length LDR primers 65°C. Primers contain dU in place of dT. Ligate with thermostable ligase at 65°C. ●
2. Extend products using one zip code primer and sequenase at 37°C. ◆
3. Digest LDR primers, LDR products and long primer extension products with Uracil N-glycosylase. ◖
4. Add fluorescent zip code primers and PCR amplify sequenase extension products using dNTPs and Taq polymerase. ◆
5. LDR product from mutant sequence generates fluorescence PCR product. PCR extension off original normal target also generates some fragments, but no fluorescence PCR product.
Tet
Fam
Bk

FIG. 21

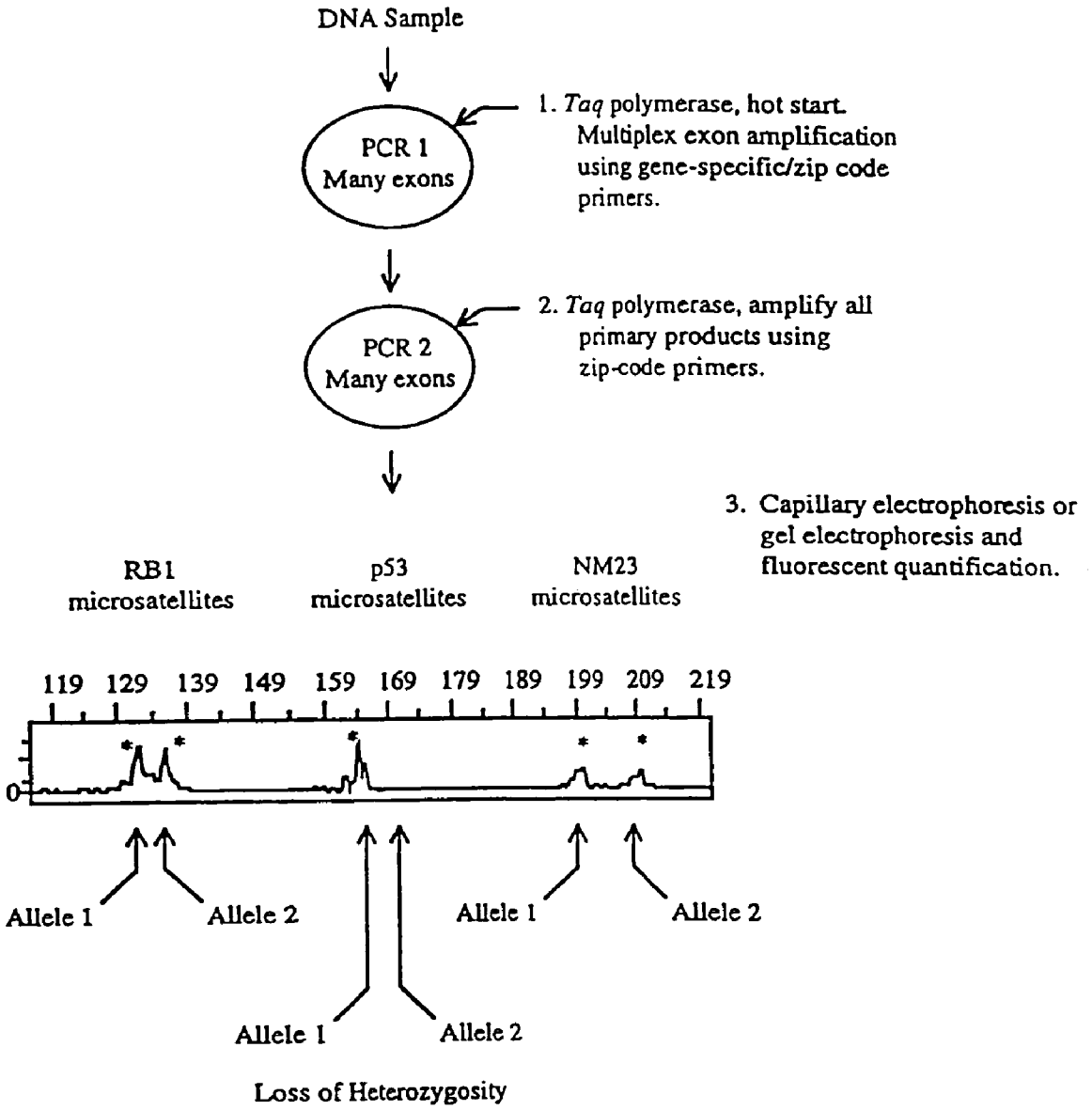

DNA Sample
PCR 1
Many exons
1. Taq polymerase, hot start. Multiplex exon amplification using gene-specific/zip code primers.
PCR 2
Many exons
2. Taq polymerase, amplify all primary products using zip-code primers.
3. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
RB1 microsatellites
p53 microsatellites
NM23 microsatellites
119 129 139 149 159 169 179 189 199 209 219
0
Allele 1
Allele 2
Allele 1
Allele 2
Allele 1
Allele 2
Loss of Heterozygosity

FIG. 22

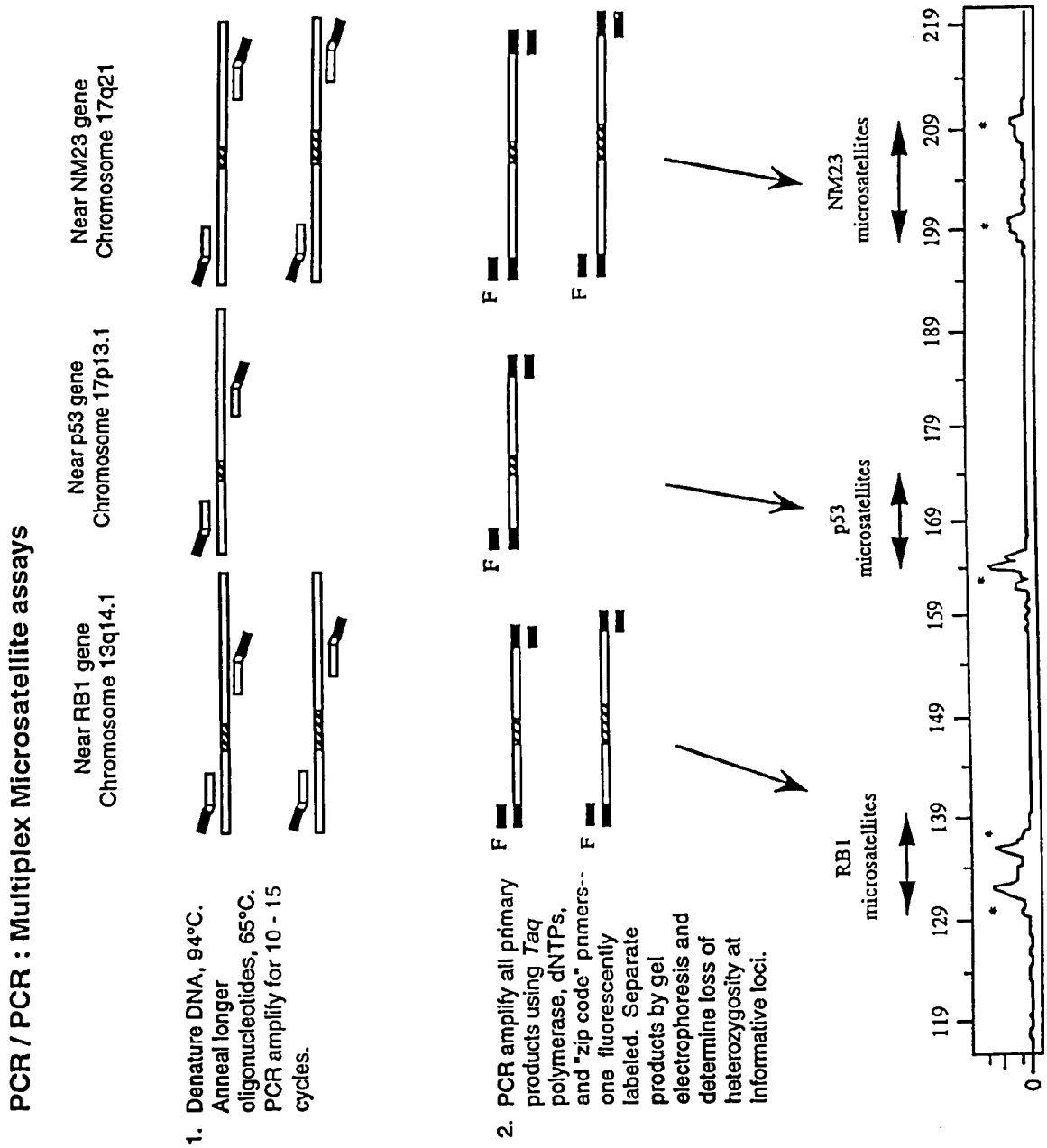

PCR / PCR : Multiplex Microsatellite assays
Near RB1 gene Chromosome 13q14.1
Near p53 gene Chromosome 17p13.1
Near NM23 gene Chromosome 17q21
1. Denature DNA, 94°C. Anneal longer oligonucleotides, 65°C. PCR amplify for 10 - 15 cycles.
2. PCR amplify all primary products using Taq polymerase, dNTPs, and "zip code" primers-- one fluorescently labeled. Separate products by gel electrophoresis and determine loss of heterozygosity at informative loci.
F
RB1 microsatellites
p53 microsatellites
NM23 microsatellites
119
129
139
149
159
169
179
189
199
209
219
0

FIG. 23

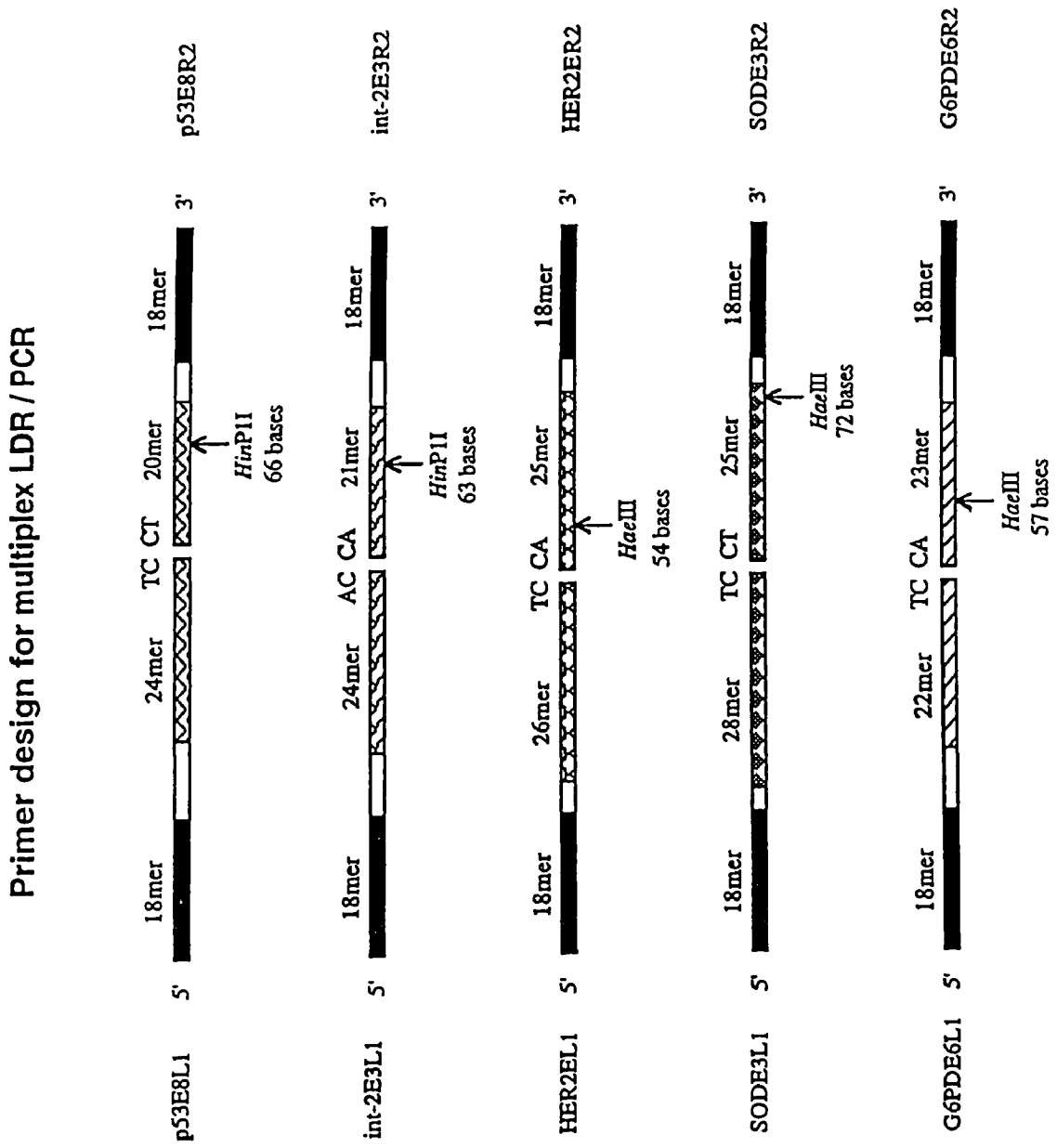

Primer design for multiplex LDR / PCR
p53E8L1
5'
18mer
24mer
TC CT
20mer
18mer
3'
p53E8R2
HinP1I
66 bases
int-2E3L1
5'
18mer
24mer
AC CA
21mer
18mer
3'
int-2E3R2
HinP1I
63 bases
HER2EL1
5'
18mer
26mer
TC CA
25mer
18mer
3'
HER2ER2
HaeIII
54 bases
SODE3L1
5'
18mer
28mer
TC CT
25mer
18mer
3'
SODE3R2
HaeIII
72 bases
G6PDE6L1
5'
18mer
22mer
TC CA
23mer
18mer
3'
G6PDE6R2
HaeIII
57 bases

FIG. 24

FIG. 25A

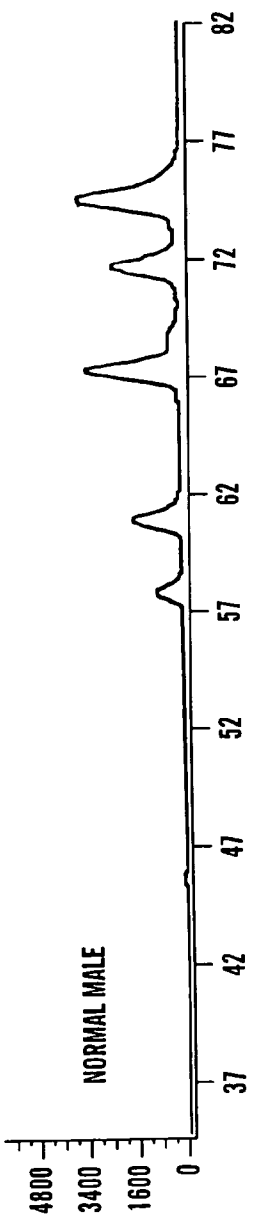


FIG. 25B

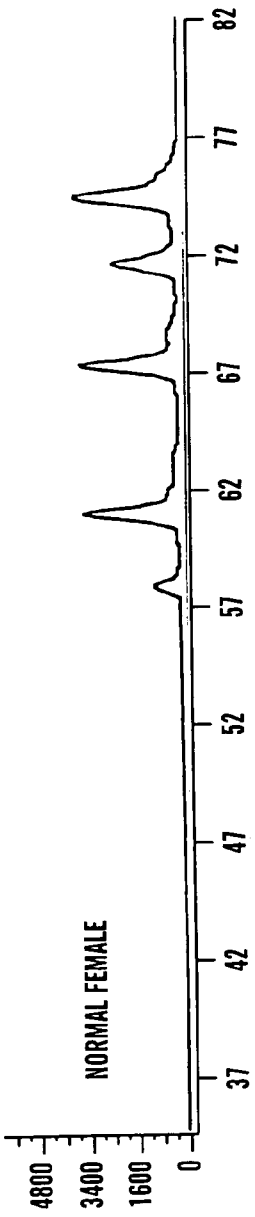


FIG. 25C

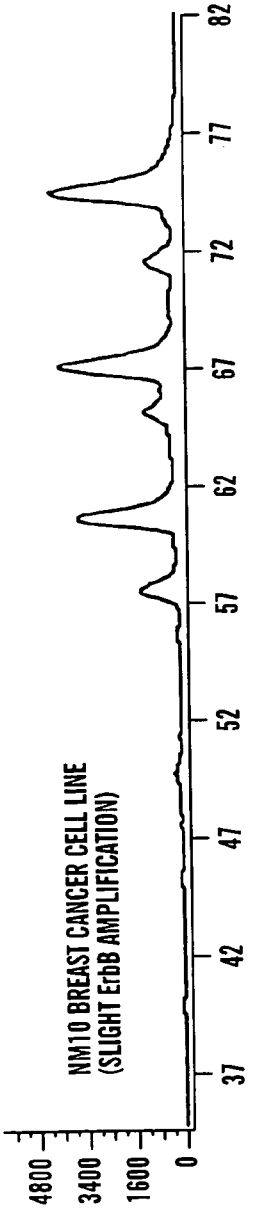


FIG. 25D

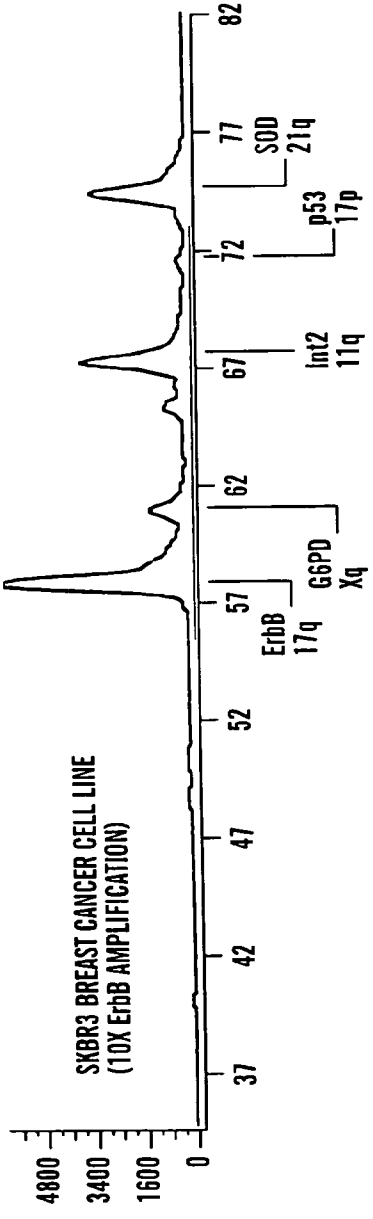


FIG. 26A

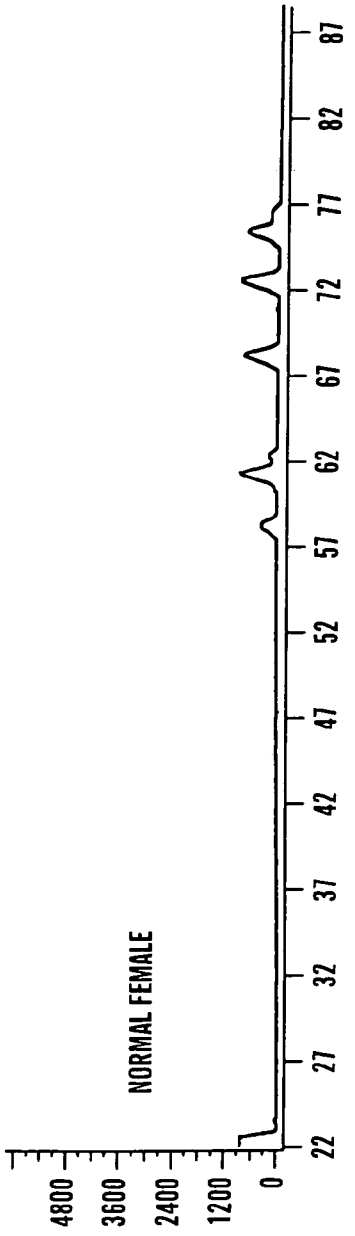


FIG. 26B

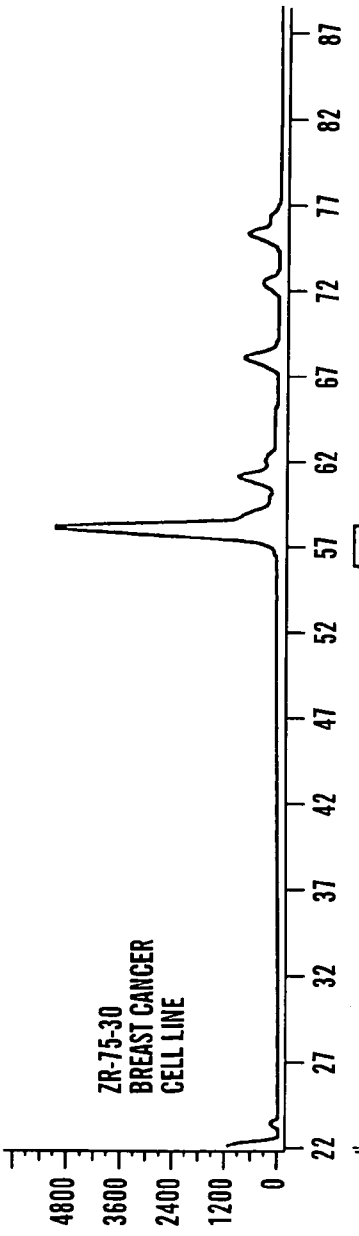


FIG. 26C

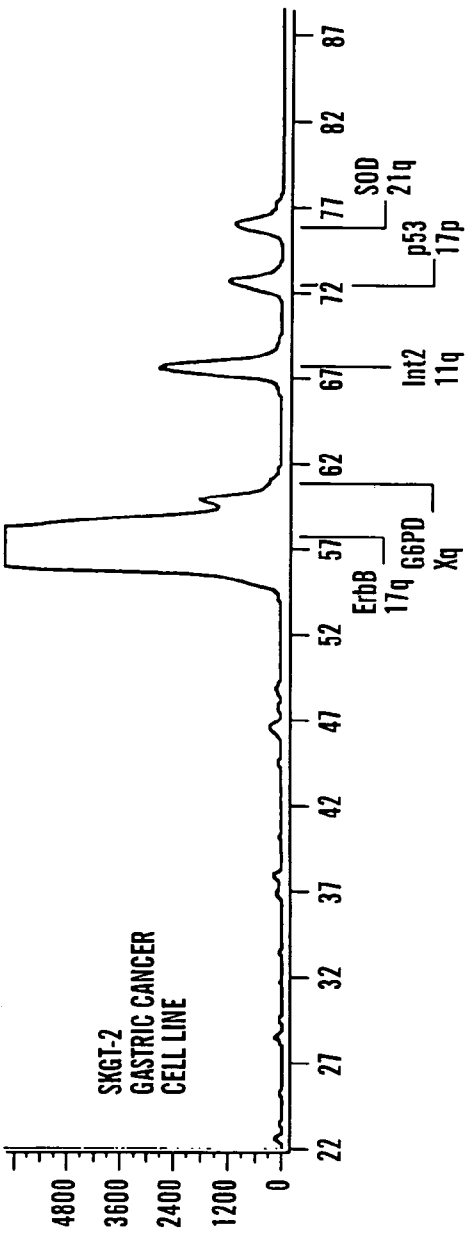


FIG. 27A

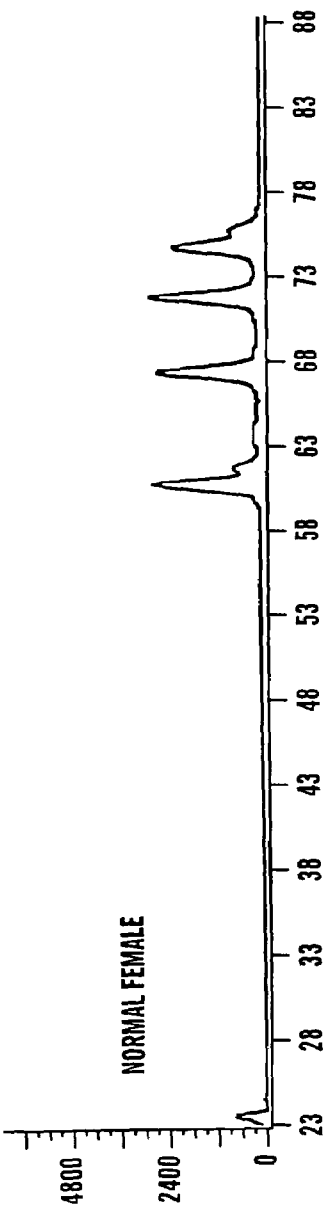


FIG. 27B

FIG. 27C

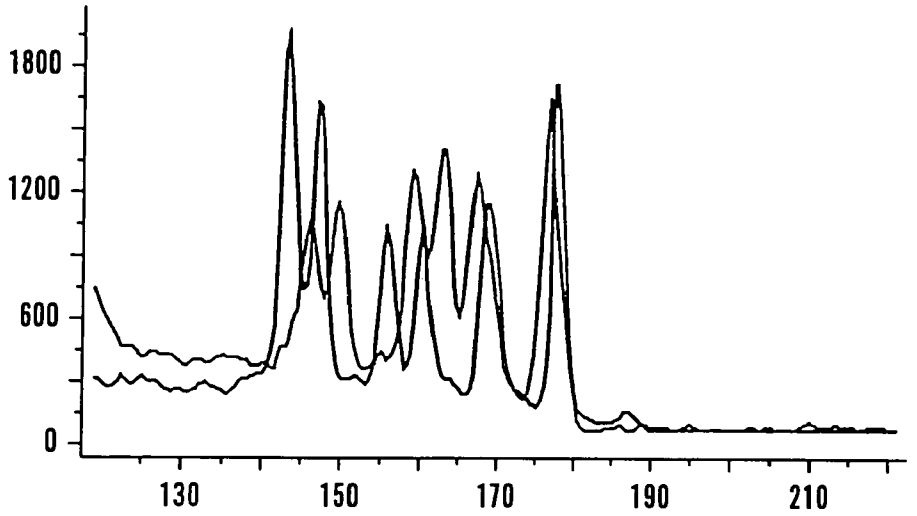


FIG. 28A

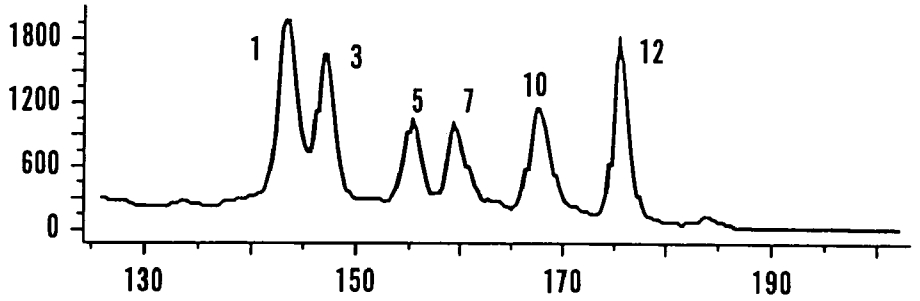


FIG. 28B

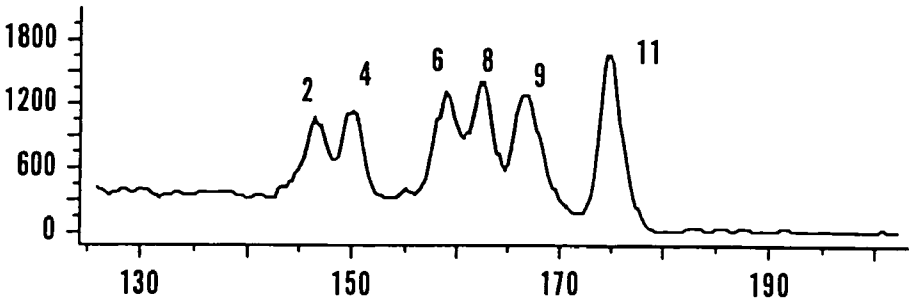


FIG. 28C

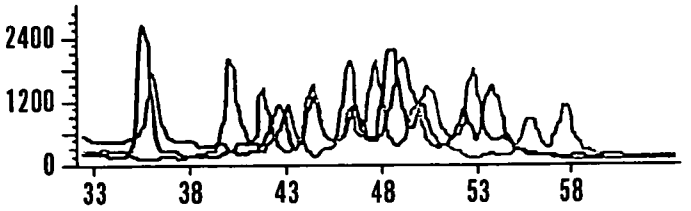


FIG. 29A

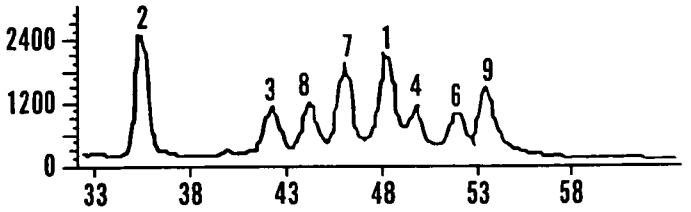


FIG. 29B

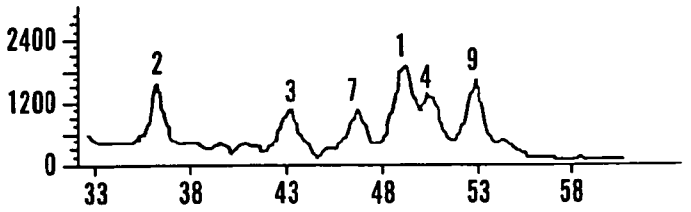


FIG. 29C

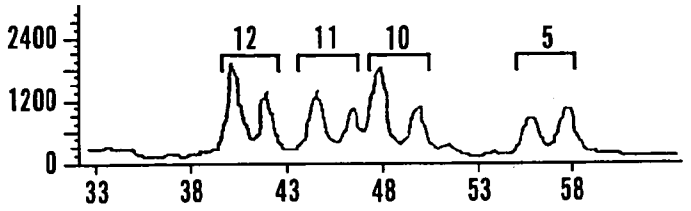


FIG. 29D

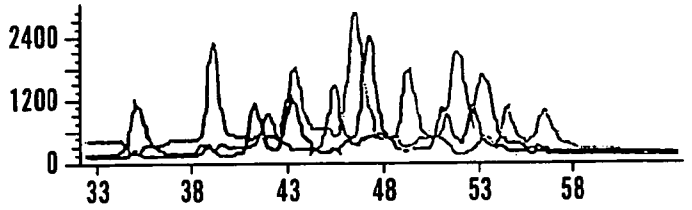


FIG. 29E

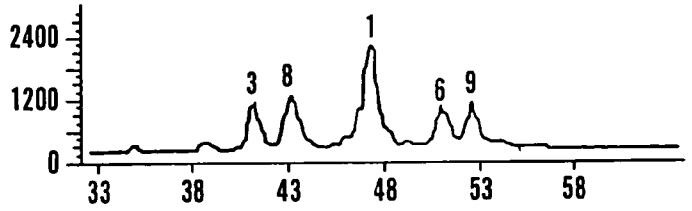


FIG. 29F

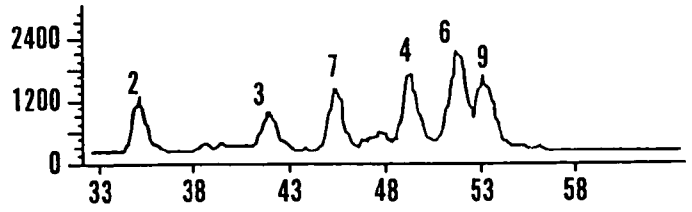


FIG. 29G

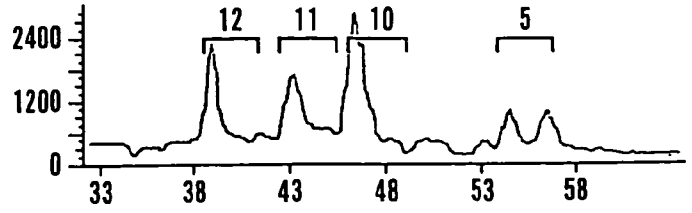


FIG. 29H

DETECTION OF NUCLEIC ACID SEQUENCE DIFFERENCES USING COUPLED LIGASE DETECTION AND POLYMERASE CHAIN REACTIONS

This application is a continuation of U.S. patent application Ser. No. 09/918,156, filed Jul. 30, 2001, now U.S. patent No. 6,797,470, issued Sep. 28, 2004, which is a continuation of U.S. patent application Ser. No. 09/440,523, filed Nov. 15, 1999, now U.S. Pat. No. 6,268,148, issued Jul. 31, 2001, which is a divisional of U.S. patent application Ser. No. 08/864,473, filed May 28, 1997, now U.S. Pat. No. 6,027,889, and claims the benefit of U.S. Provisional Patent Application Ser. No. 60/018,532, filed May 29, 1996, which are all hereby incorporated by reference in their entirety.

This invention was developed with government funding under National Institutes of Health Grant No. GM41337-06. The U.S. Government may retain certain rights.

FIELD OF THE INVENTION

The present invention relates to the detection of nucleic acid sequence differences using coupled ligase detection reaction ("LDR") and polymerase chain reaction ("PCR"). One aspect of the present invention involves use of a ligase detection reaction coupled to a polymerase chain reaction. Another aspect of the present invention relates to the use of a primary polymerase chain reaction coupled to a secondary polymerase chain reaction coupled to a ligase detection reaction. A third aspect of the present invention involves a primary polymerase chain reaction coupled to a secondary polymerase chain reaction.

BACKGROUND OF THE INVENTION

Multiplex Detection

Large-scale multiplex analysis of highly polymorphic loci is needed for practical identification of individuals, e.g., for paternity testing and in forensic science (Reynolds et al., Anal. Chem., 63:2–15 (1991)), for organ-transplant donor-recipient matching (Buyse et al., *Tissue Antigens,* 41:1–14 (1993) and Gyllensten et al., *PCR Meth. Appl,* 1:91–98 (1991)), for genetic disease diagnosis, prognosis, and prenatal counseling (Chamberlain et al., *Nucleic Acids Res.,* 16:11141–11156 (1988) and L. C. Tsui, *Human Mutat.,* 1:197–203 (1992)), and the study of oncogenic mutations (Hollstein et al., *Science,* 253:49–53 (1991)). In addition, the cost-effectiveness of infectious disease diagnosis by nucleic acid analysis varies directly with the multiplex scale in panel testing. Many of these applications depend on the discrimination of single-base differences at a multiplicity of sometimes closely spaced loci.

A variety of DNA hybridization techniques are available for detecting the presence of one or more selected polynucleotide sequences in a sample containing a large number of sequence regions. In a simple method, which relies on fragment capture and labeling, a fragment containing a selected sequence is captured by hybridization to an immobilized probe. The captured fragment can be labeled by hybridization to a second probe which contains a detectable reporter moiety.

Another widely used method is Southern blotting. In this method, a mixture of DNA fragments in a sample is fractionated by gel electrophoresis, then fixed on a nitrocellulose filter. By reacting the filter with one or more labeled probes under hybridization conditions, the presence of bands containing the probe sequences can be identified. The method is especially useful for identifying fragments in a restriction-enzyme DNA digest which contains a given probe sequence and for analyzing restriction-fragment length polymorphisms ("RFLPs").

Another approach to detecting the presence of a given sequence or sequences in a polynucleotide sample involves selective amplification of the sequence(s) by polymerase chain reaction. U.S. Pat. No. 4,683,202 to Mullis, et al. and R. K. Saiki, et al., *Science* 230:1350 (1985). In this method, primers complementary to opposite end portions of the selected sequence(s) are used to promote, in conjunction with thermal cycling, successive rounds of primer-initiated replication. The amplified sequence(s) may be readily identified by a variety of techniques. This approach is particularly useful for detecting the presence of low-copy sequences in a polynucleotide-containing sample, e.g., for detecting pathogen sequences in a body-fluid sample.

More recently, methods of identifying known target sequences by probe ligation methods have been reported. U.S. Pat. No. 4,883,750 to N. M. Whiteley, et al., D. Y. Wu, et al., *Genomics* 4:560 (1989), U. Landegren, et al., *Science* 241:1077 (1988), and E. Winn-Deen, et al., *Clin. Chem.* 37:1522 (1991). In one approach, known as oligonucleotide ligation assay ("OLA"), two probes or probe elements which span a target region of interest are hybridized to the target region. Where the probe elements basepair with adjacent target bases, the confronting ends of the probe elements can be joined by ligation, e.g., by treatment with ligase. The ligated probe element is then assayed, evidencing the presence of the target sequence.

In a modification of this approach, the ligated probe elements act as a template for a pair of complementary probe elements. With continued cycles of denaturation, hybridization, and ligation in the presence of pairs of probe elements, the target sequence is amplified linearly, allowing very small amounts of target sequence to be detected and/or amplified. This approach is referred to as ligase detection reaction. When two complementary pairs of probe elements are utilized, the process is referred to as the ligase chain reaction which achieves exponential amplification of target sequences. F. Barany, "Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase," *Proc. Nat'l Acad. Sci USA,* 88:189–93 (1991) and F. Barany, "The Ligase Chain Reaction (LCR) in a PCR World," *PCR Methods and Applications,* 1:5–16 (1991).

Another scheme for multiplex detection of nucleic acid sequence differences is disclosed in U.S. Pat. No. 5,470,705 to Grossman et. al. where sequence-specific probes, having a detectable label and a distinctive ratio of charge/translational frictional drag, can be hybridized to a target and ligated together. This technique was used in Grossman, et. al., "High-density Multiplex Detection of Nucleic Acid Sequences: Oligonucleotide Ligation Assay and Sequence-coded Separation," *Nucl. Acids Res.* 22(21):4527–34 (1994) for the large scale multiplex analysis of the cystic fibrosis transmembrane regulator gene.

Jou, et. al., "Deletion Detection in Dystrophia Gene by Multiplex Gap Ligase Chain Reaction and Immunochromatographic Strip Technology," *Human Mutation* 5:86–93 (1995) relates to the use of a so called "gap ligase chain reaction" process to amplify simultaneously selected regions of multiple exons with the amplified products being read on an immunochromatographic strip having antibodies specific to the different haptens on the probes for each exon.

There is a growing need (e.g., in the field of genetic screening) for methods useful in detecting the presence or

absence of each of a large number of sequences in a target polynucleotide. For example, as many as 400 different mutations have been associated with cystic fibrosis. In screening for genetic predisposition to this disease, it is optimal to test all of the possible different gene sequence mutations in the subject's genomic DNA, in order to make a positive identification of "cystic fibrosis". It would be ideal to test for the presence or absence of all of the possible mutation sites in a single assay. However, the prior-art methods described above are not readily adaptable for use in detecting multiple selected sequences in a convenient, automated single-assay format.

Solid-phase hybridization assays require multiple liquid-handling steps, and some incubation and wash temperatures must be carefully controlled to keep-the-stringency needed for single-nucleotide mismatch discrimination. Multiplexing of this approach has proven difficult as optimal hybridization conditions vary greatly among probe sequences.

Developing a multiplex PCR process that yields equivalent amounts of each PCR product can be difficult and laborious. This is due to variations in the annealing rates of the primers in the reaction as well as varying polymerase extension rates for each sequence at a given Mg2+ concentration. Typically, primer, Mg2+, and salt concentrations, along with annealing temperatures are adjusted in an effort to balance primer annealing rates and polymerase extension rates in the reaction. Unfortunately, as each new primer set is added to the reaction, the number of potential amplicons and primer dimers which could form increase exponentially. Thus, with each added primer set, it becomes increasingly more difficult and time consuming to work out conditions that yield relatively equal amounts of each of the correct products.

Allele-specific PCR products generally have the same size, and an assay result is scored by the presence or absence of the product band(s) in the gel lane associated with each reaction tube. Gibbs et al., *Nucleic Acids Res.,* 17:2437–2448 (1989). This approach requires splitting the test sample among multiple reaction tubes with different primer combinations, multiplying assay cost. PCR has also discriminated alleles by attaching different fluorescent dyes to competing allelic primers in a single reaction tube (F. F. Chehab, et al., *Proc. Natl. Acad. Sci. USA,* 86:9178–9182 (1989)), but this route to multiplex analysis is limited in scale by the relatively few dyes which can be spectrally resolved in an economical manner with existing instrumentation and dye chemistry. The incorporation of bases modified with bulky side chains can be used to differentiate allelic PCR products by their electrophoretic mobility, but this method is limited by the successful incorporation of these modified bases by polymerase, and by the ability of electrophoresis to resolve relatively large PCR products which differ in size by only one of these groups. Livak et al., *Nucleic Acids Res.,* 20:4831–4837 (1989). Each PCR product is used to look for only a single mutation, making multiplexing difficult.

Ligation of allele-specific probes generally has used solid-phase capture (U. Landegren et al., *Science,* 241:1077–1080 (1988); Nickerson et al., *Proc. Natl. Acad. Sci. USA,* 87:8923–8927 (1990)) or size-dependent separation (D. Y. Wu, et al., *Genomics,* 4:560–569 (1989) and F. Barany, *Proc. Natl. Acad. Sci.,* 88:189–193 (1991)) to resolve the allelic signals, the latter method being limited in multiplex scale by the narrow size range of ligation probes. Further, in a multiplex format, the ligase detection reaction alone cannot make enough product to detect and quantify small amounts of target sequences. The gap ligase chain reaction process requires an additional step—polymerase extension. The use of probes with distinctive ratios of charge/translational frictional drag for a more complex multiplex will either require longer electrophoresis times or the use of an alternate form of detection.

The need thus remains for a rapid single assay format to detect the presence or absence of multiple selected sequences in a polynucleotide sample.

Microsatellite Analysis

Tandem repeat DNA sequences known as microsatellites represent a very common and highly polymorphic class of genetic elements within the human genome. These microsatellite markers containing small repeat sequences have been used for primary gene mapping and linkage analysis. Weber, J. L. et al., *Am. J. Hum. Genet.* 44: 388–396 (1989); Weissenbach, J. et al., *Nature* (*London*) 359: 794–800 (1992). PCR amplification of these repeats allows rapid assessment for loss of heterozygosity and can greatly simplify procedures for mapping tumor suppressor genes. Ruppert, J. M., et al., *Cancer Res.* 53: 5093–94 (1993); van der Riet, et al., *Cancer Res.* 54: 1156–58(1994); Nawroz, H., et al., *Cancer Res.* 54: 1152–55(1994); Cairns, P., et al., *Cancer Res.* 54: 1422–24 (1994). More recently, they have been used to identify specific mutations in certain inherited disorders including Huntington disease, fragile X syndrome, myotonic dystrophy, spinocerebellar ataxia type I, spinobulbar muscular atrophy, and hereditary dentatorubral-pallidoluysian atrophy. The Huntington's Disease Collaborative Research Group *Cell* 72: 971–83 (1993); Kremer, E. J., et al., *Science* 252: 1711–14 (1991); Imbert, G., et al., *Nat. Genet.* 4: 72–76 (1993); Orr, H. T., et al., *Nat. Genet.* 4: 221–226 (1993); Biancalana, V., et al., *Hum. Mol. Genet.* 1: 255–258 (1992); Chung, M.-Y., et al., *Nat. Genet.* 5: 254–258 (1993); Koide, R., et al., *Nat. Genet.* 6: 9–13 (1994). These inherited disorders appear to arise from the expansion of trinucleotide repeat units within susceptible genes. A more widespread microsatellite instability, demonstrated by expansion or deletion of repeat elements in neoplastic tissues, was first reported in colorectal tumors. Peinado, M. A., et al. *Proc. Natl. Acad. Sci. USA* 89: 10065–69 (1992); Ionov, Y., *Nature* (*London*) 363: 558–61 (1993); Thibodeau, S. N., et al., *Science* 260: 816–819 (1993) and later in several other tumor types (Risinger, J. I., *Cancer Res.* 53: 5100–03 (1993); Han, H.-J., et al., *Cancer Res.* 53: 5087–89 (1993); Peltomäki, P., *Cancer Res.* 53: 5853–55 (1993); Gonzalez-Zulueta, M., et al., *Cancer Res.* 53: 5620–23 (1993); Merlo, A., et al., *Cancer Res.* 54: 2098–2101 (1994)). In hereditary nonpolyposis colorectal carcinoma patients, this genetic instability is apparently due to inherited and somatic mutations in mismatch repair genes. Leach, F., et al., *Cell* 75: 1215–1225 (1993); Fishel, R., et al., *Cell* 75: 1027–38 (1993); Papadopoulos, N., et al., *Science* 263: 1625–29 (1994); Bronner, C. E., et al., *Nature* (*London*) 368: 258–61 (1994).

PCR is commonly used for microsatellite analysis in identifying both the appearance of new polymorphisms and the loss of heterozygosity in cancer detection. L. Mao, et. al., "Microsatellite Alterations as Clonal Markers for the Detection of Human Cancer," *Proc. Nat'l Acad. Sci USA* 91(21): 9871–75 (1994); L. Mao, et. al., "Molecular Detection of Primary Bladder Cancer by Microsatellite Analysis," *Science* 271:659–62 (1996); D. Radford, et. al., "Allelotyping of Ductal Carcinoma in situ of the Breast: Detection of Loci on 8p, 13q, 161, 17p and 17q," *Cancer Res.* 55(15): 3399–05 (1995). In using PCR for such purposes, each PCR reaction is run individually and separated on a sequencing gel.

Although these references demonstrate that PCR has application to diagnosis and prognosis of certain cancers, this type of analysis is deficient, because it does not permit a high throughput and requires size separation. In addition, there are problems with PCR slippage, causing researchers to shifi to tri-, tetra-, and higher nucleotide repeat units, making cancer detection more difficult.

Microsatellite markers have also been used for colon cancer detection (L. Cawkwell, et. al., "Frequency of Allele Loss of DCC, p53, RB1, WT1, NF1, NM23, and APC/MCC in Colorectal Cancer Assayed by Fluorescent Multiplex Polymerase Chain Reaction," *Br. J. Cancer* 70(5): 813–18 (1994)) and for genome mapping (P. Reed, et. al., "Chromosome-specific Microsatellite Sets for Fluorescent-Based, Semi-Automated Genome Mapping," *Nat. Genet.* 7(3): 390–95 (1994)). However, the key to such multiplex processes is the ability to perform them in a single reaction tube. Conventional multiplex microsatellite marker approaches require careful attention to primer concentrations and amplification conditions. Although PCR products can be pooled in sets, this requires a prerun on agarose gels to insure that the mixture has about equal amounts of DNA in each band.

Human Identification

PCR has also been used for human identification, such as paternity testing, criminal investigations, and military personnel identification. A. Syvanen et. al., "Identification of Individuals by Analysis of Biallelic DNA Markers, Using PCR and Solid-Phase Mini-Sequencing" *Am. J. Hum. Genet.* 52(1): 46–59 (1993) describes a mini-sequencing approach to human identification. The technique requires PCR amplification of individual markers with at most 4 PCR reactions being carried out at a time in a single PCR tube. Mini-sequencing is carried out to determine individual polymorphisms.

Coupled Processes

G. Deng, et. al., "An Improved Method of Competitive PCR for Quantitation of Gene Copy Number," *Nucl. Acids Res.* 21: 4848–49 (1993) describes a competitive PCR process. Here, two PCR steps are utilized with different sets of primers being used for each gene and its equivalent standard.

T. Msuih, et. al., "Novel, Ligation-Dependent PCR Assay for Detection of Hepatitis C. Virus in Serum," *J. Clin Microbio.* 34: 501–07 (1996) and Y. Park, et. al., "Detection of HCV RNA Using Ligation-Dependent Polymerase Chain Reaction in Formalin-Fixed Paraffin-Embedded Liver Tissue" (submitted) describe the use of a LDR/PCR process in work with RNA.

SUMMARY OF THE INVENTION

The present invention is directed to the detection of nucleic acid sequence differences using coupled LDR and PCR processes. The present invention can be carried out in one of the following 3 embodiments: (1) LDR coupled to PCR; (2) primary PCR coupled to secondary PCR coupled to LDR; and (3) primary PCR coupled to secondary PCR. Each of these embodiments have particular applicability in detecting certain characteristics. However, each requires the use of coupled reactions for multiplex detection of nucleic acid sequence differences where oligonucleotides from an early phase of each process contain sequences which may be used by oligonucleotides from a later phase of the process.

I. Primary PCR/Secondary PCR/LDR Process

One aspect of the present invention relates to a method for identifying two or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. This method involves a first polymerase chain reaction phase, a second polymerase chain reaction phase, and a ligase detection reaction phase. This process involves analyzing a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences.

In the first polymerase chain reaction phase, one or more primary oligonucleotide primer groups are provided. Each group comprises one or more primary oligonucleotide primer sets with each set having a first nucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion, and a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion. The first oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion and the second oligonucleotide primers of each set in the same group contain the same 5' upstream primer-specific portion. The oligonucleotide primers in a particular set are suitable for hybridization on complementary strands of a corresponding target nucleotide sequence to permit formation of a polymerase chain reaction product. However, there is a mismatch which interferes with formation of such a polymerase chain reaction product when the primary oligonucleotide primers hybridize to any other nucleotide sequence in the sample. The polymerase chain reaction products in a particular set may be distinguished from other polymerase chain reaction products in the same group or groups. The primary oligonucleotide primers, the sample, and the polymerase are blended to form a primary polymerase chain reaction mixture.

The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as substantially described above. During hybridization, target-specific portions of the primary oligonucleotide primers hybridize to the target nucleotide sequences. The extension treatment causes hybridized primary oligonucleotide primers to be extended to form primary extension products complementary to the target nucleotide sequence to which the primary oligonucleotide primers are hybridized.

Although the upstream secondary primer-specific portions of a primary oligonucleotide primer set are not present on the target DNA, their sequences are copied by the second and subsequent cycles of the primary polymerase chain reaction phase. As a result, the primary extension products produced after the second cycle have the secondary primer-specific portions on their 5' ends and the complement of primer-specific portion on their 3' ends.

Next, there is a second polymerase chain reaction phase. This phase involves providing one or a plurality of secondary oligonucleotide primer sets. Each set has a first secondary oligonucleotide primer containing the same sequence as the 5' upstream portion of the first primary oligonucleotide primer, and a second secondary oligonucleotide primer containing the same sequence as the 5' upstream portion of the second primary oligonucleotide primer from the same primary oligonucleotide primer set as the first primary oligonucleotide complementary to the first secondary primer. A set of secondary oligonucleotide primers may be used to amplify all of the primary extension products in a

given group. The secondary oligonucleotide primers are blended with the primary extension products and the polymerase to form a secondary polymerase chain reaction mixture.

The secondary polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles having a denaturation treatment, a hybridization treatment, and an extension treatment, as substantially set forth above. During the hybridization treatment, the secondary oligonucleotide primers hybridize to the complementary sequences present on the primary extension products but not to the original target sequence. The extension treatment causes the hybridized secondary oligonucleotide primers to be extended to form secondary extension products complementary to the primary extension products.

The last phase of this aspect of the present invention involves a ligase detection reaction process. Here, a plurality of oligonucleotide probe sets are provided where each set has a first oligonucleotide probe, having a secondary extension product-specific portion and a detectable reporter label, and a second oligonucleotide probe, having a secondary extension product-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a complementary secondary extension product-specific portion. However, there is a mismatch which interferes with such ligation when the oligonucleotide probes are hybridized to any other nucleotide sequence present in the sample. The ligation product of oligonucleotide probes in a particular set may be distinguished from either probe or other ligation products. The plurality of oligonucleotide probe sets, the secondary extension products, and a ligase are blended to form a ligase detection reaction mixture.

The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles having a denaturation treatment and hybridization treatment substantially as described above. In the hybridization treatment, the oligonucleotide probe sets hybridize at adjacent positions in a base-specific manner to the respective secondary extension products if present. As a result, adjacent probes ligate to one another to form a ligation product sequence containing the detectable reporter label and the secondary extension product-specific portions connected together. The oligonucleotide probe sets may hybridize to nucleotide sequences other than their respective complementary secondary extension products but do not ligate together due to the presence of one or more mismatches and individually separate during the denaturation treatment. Following the ligase detection reaction cycles, the reporter labels of the ligation product sequences are detected which indicates the presence of one or more target nucleotide sequences in the sample.

The primary PCR/secondary PCR/LDR process of the present invention provides significant advantages over the use of PCR alone in the multiplex detection of single nucleotide and tandem repeat polymorphisms.

As noted above, the use of PCR alone requires heavy optimization of operating conditions in order to conduct multiplex detection procedures. Moreover, the quantity of oligonucleotide primers must be increased to detect greater numbers of target nucleotide sequences. However, as this occurs, the probability of target independent reactions (e.g., the primer-dimer effect) increases. In addition, the mutations must be known, false positives may be generated by polymerase extension off of normal template, closely-clustered sites due to interference of overlapping primers cannot undergo multiplex detection, single base or small insertions and deletions in small repeat sequences cannot be detected, and quantification of mutant DNA in high background of normal DNA is difficult. As a result, the number of target nucleotide sequences detected in a single multiplex PCR process is limited.

Direct sequencing requires enrichment of mutant samples in order to correct sequences, requires multiple reactions for large genes containing many exons, requires electrophoretic separation of products, is time consuming, and cannot be used to detect mutant DNA in less than 5% of background of normal DNA. When mini-sequencing, the mutation must be known, closely-clustered sites due to interference of overlapping primers cannot undergo multiplex detection, single base or small insertions and deletions in small repeat sequences cannot be detected, and four separate reactions are required. For allele-specific oligonucleotide hybridization ("ASO"), the mutation must be known, hybridization and washing conditions must be known, cross-reactivity is difficult to prevent, closely-clustered sites due to interference of overlapping primers cannot undergo multiplex detection, and mutant DNA cannot be detected in less than 5% of background of normal DNA. Primer-mediated RFLP requires electrophoretic separation to distinguish mutant from normal DNA, is of limited applicability to sites that may be converted into a restriction site, requires additional analysis to determine the nature of the mutation, and is difficult to use where the mutant DNA is in a high background of normal DNA. Single strand conformational polymorphism analysis ("SSCP") requires electrophoretic separation to distinguish mutant conformer from normal conformer, misses 30% of possible mutations, requires additional analysis to determine the nature of the mutation, and cannot distinguish mutations from silent polymorphisms. With dideoxynucleotide finger printing ("ddF"), it is difficult to detect mutations in a high background of normal DNA, electrophoretic separation is required to distinguish mutant conformer from normal conformer, additional analysis must be used to determine the nature of the mutation, and mutations cannot be distinguished from silent polymorphisms. Denaturing gradient gel electrophoresis ("DGGE") must electrophoretically separate mutant conformer from normal conformer, misses 30% of possible mutations, requires additional analysis to determine the nature of the mutation, cannot distinguish mutations from silent polymorphisms, and imposes technical challenges to reproducing previously achieved results. RNase mismatch cleavage requires additional analysis to determine the nature of the mutation, requires analysis of both strands to exclude RNase-resistant mismatches, and imposes difficulty in detecting mutations in a high background of normal DNA. Chemical mismatch cleavage cannot detect mutant DNA in less than 5% of background of normal DNA, and requires an analysis of both strands to detect all mutations. For T4 Endo VII mismatch cleavage, additional analysis is needed to determine the nature of the mutation, mutations cannot be distinguished from silent polymorphisms, endonuclease cleaves control DNA which necessitates careful interpretation of results, and it is difficult to detect mutations in a high background of normal DNA.

These problems are avoided in the primary PCR/secondary PCR/LDR process of the present invention which combines the sensitivity of PCR with the specificity of LDR. The primary PCR phase produces primary extension products with a secondary primer-specific portion. This initial phase is carried out under conditions effective to maximize production of primary extension products without obtaining the adverse effects sometimes achieved in a PCR-only process. In particular, the primary PCR phase of the present invention

is carried out with 15 to 20 PCR cycles and utilizes less primer than would be utilized in a PCR-only process. The primary PCR phase of the present invention produces extension products in a varied and unpredictable way, because some target nucleotide sequences will be amplified well, while others will not. However, in the secondary PCR phase, all of the primary extension products are amplified approximately equally, because they all have the same secondary primer-specific portions. Target nucleotide sequences originally present in the sample will not be amplified by the secondary PCR phase, because such sequences do not contain a secondary primer-specific portion. As a result, the primary PCR/secondary PCR/LDR process of the present invention is able to achieve multiplex detection of hundreds of nucleotide sequence differences in a single tube without undue customization of operating conditions for each particular sample being analyzed. Since the selection of mutant sequences is mediated by LDR rather than PCR, the primary PCR/secondary PCR/LDR process is less susceptible to false-positive signal generation. In addition, the primary PCR/secondary PCR/LDR process allows detection of closely-clustered mutations, detection of single base or small insertions and deletions in small repeat sequences, quantitative detection of less than 1% mutations in high background of normal DNA, and detection of ligation product sequences using addressable arrays. The only significant requirements are that the mutations be known and that a multitude of oligonucleotides be synthesized.

The ability to detect single nucleotide and tandem repeat polymorphisms is particularly important for forensic DNA identification and diagnosis of genetic diseases.

II. LDR/PCR Process

A second aspect of the present invention relates to a method for identifying one or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. This method has a ligase detection reaction phase followed by a polymerase chain reaction phase. This method involves providing a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences.

In the ligase detection reaction phase, one or more oligonucleotide probe sets are provided. Each set has a first oligonucleotide probe, having a target-specific portion and a 5' upstream primer-specific portion, and a second oligonucleotide probe, having a target-specific portion and a 3' downstream primer-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target nucleotide sequence. However, there is a mismatch which interferes with such ligation when they are hybridized to any other nucleotide sequence present in the sample. The sample, the plurality of oligonucleotide probe sets, and a ligase are blended together to form a ligase detection reaction mixture.

The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles. These cycles include a denaturation treatment and a hybridization treatment. In the denaturation treatment, any hybridized oligonucleotides are separated from the target nucleotide sequences. The hybridization treatment causes the oligonucleotide probe sets to hybridize at adjacent positions in a base-specific manner to their respective target nucleotide sequences if present in the sample. Once hybridized, the oligonucleotide probe sets ligate to one another to form a ligation product sequence. This product contains the 5' upstream primer-specific portion, the target-specific portions connected together, and the 3' downstream primer-specific portion. The ligation product sequence for each set is distinguishable from other nucleic acids in the ligase detection reaction mixture. The oligonucleotide probe sets hybridized to nucleotide sequences in the sample other than their respective target nucleotide sequences but do not ligate together due to a presence of one or more mismatches and individually separate during the subsequent denaturation treatment.

In the polymerase chain reaction, one or a plurality of oligonucleotide primer sets are provided. Each set has an upstream primer containing the same sequence as the 5' upstream primer-specific portion of the ligation product sequence and a downstream primer complementary to the 3' downstream primer-specific portion of the ligation product sequence, where one primer has a detectable reporter label. The ligase detection reaction mixture is blended with the one or a plurality of oligonucleotide primer sets and the polymerase to form a polymerase chain reaction mixture.

The polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles which include a denaturation treatment, a hybridization treatment, and an extension treatment. During the denaturation treatment, hybridized nucleic acid sequences are separated. The hybridization treatment causes primers to hybridize to their complementary primer-specific portions of the ligation product sequence. During the extension treatment, hybridized primers are extended to form extension products complementary to the sequences to which the primers are hybridized. In a first cycle of the polymerase chain reaction phase, the downstream primer hybridizes to the 3' downstream primer-specific portion of the ligation product sequence and is extended to form an extension product complementary to the ligation product sequence. In subsequent cycles the upstream primer hybridizes to the 5' upstream primer-specific portion of the extension product complementary to the ligation product sequence and the downstream primer hybridizes to the 3' downstream portion of the ligation product sequence.

Following the polymerase chain reaction phase of this process, the reporter labels are detected and the extension products are distinguished to indicate the presence of one or more target nucleotide sequences in the sample.

One embodiment of the LDR/PCR process of the present invention achieves improved results over the use of LDR alone in measuring the number of gene copies in a cell (i.e. gene dosage). When LDR alone is utilized, it is difficult to produce sufficient target copies which are needed ultimately to quantify a plurality of genes.

In another embodiment of the LDR/PCR process of the present invention, the LDR phase ligation product sequences are produced in a ratio proportional to the ratio of the genes from which they were derived within the sample. By incorporation of the same primer-specific portions in the oligonucleotide probes for the LDR phase, the PCR phase amplifies ligation product sequences to the same degree so that their proportionality is maintained. Target sequences originally found in the sample being analyzed are not amplified by the PCR phase, because such target sequences do not contain PCR primer-specific portions. In addition, since only the oligonucleotide primers for the PCR phase have reporter labels, only extension products with those labels will be detected.

Determination of variation in gene dosage is important in a number of biomedical applications.

Males differ in gene dosage from females for those genes located on the X chromosome, women having two copies while men have one copy. Women may be carriers of deletions along the X chromosome. If the deleted region of the X chromosome included one or more genes, then the woman has only one copy of these genes in the corresponding, non-deleted area of the other X chromosome. The result (having one copy of an X-linked gene) is similar to the situation in male cells and is usually tolerated without manifestations of an inherited disorder. However, if the woman's-son inherits her deleted X chromosome, he will have no copies of the genes in the deletion region and suffer from the X-linked disorder related to the absence of the gene. The detection of chromosomal deletions, therefore, is one application of the LDR/PCR process of the present invention.

Congenital chromosomal disorders occur when a fertilized egg has an abnormal compliment of chromosomes. The most common congenital chromosomal disorder is Down Syndrome, which occurs when there is an additional chromosome 21 in each cell, designated 47,XX+21 or 47,XY+21. The LDR/PCR process of the present invention can be designed to identify congenital chromosomal disorders.

The LDR/PCR process of the present invention is also useful for distinguishing polymorphisms in mono-nucleotide and di-nucleotide repeat sequences. It will also be useful in distinguishing minor populations of cells containing unique polymorphisms for detection of clonality.

The LDR/PCR process can be used with both gel and non-gel (i.e. DNA array) technologies. It allows multiplex analysis of many gene amplifications and deletions simultaneously, allows quantitative analysis, and does not require an external standard. The only relatively minor challenge presented by the LDR/PCR process is that it is difficult to use in determining the boundaries of large deletions in chromosomes.

By contrast, microsatellite marker analysis cannot be used to detect small regions that are deleted or amplified, is not compatible with simultaneous detection of amplified regions, and depends on the availability of informative markers. Competitive PCR (i.e. differential PCR) cannot be used in a multiplex format to detect several deletions and amplifications simultaneously, and is not particularly accurate. Southern hybridization is time consuming, labor intensive, is not amenable to multiplexing due to the need for multiple steps for each probe tested, and requires large quantities of DNA. Fluorescent in situ hybridization ("FISH") requires specialized expertise, is time consuming, and requires large probes to analyze for suspected deleted or amplified regions. Thus, the LDR/PCR process of the present invention constitutes a significant advance over prior processes.

III. Primary PCR/Secondary PCR Process

A third aspect of the present invention also involves a method for identifying two or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in one or more target nucleotide sequences. This method involves subjecting a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences to two successive polymerase chain reaction phases.

For the first polymerase chain reaction phase, one or more primary oligonucleotide primer groups are provided where each group comprises two or more primary oligonucleotide primer sets. Each set has a first oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primary-specific portion, and a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion. The first oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion and the second oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion. The oligonucleotide primers in a particular set are suitable for hybridization on complementary strands of a corresponding target nucleotide sequence to permit formation of a polymerase chain reaction product. However, there is a mismatch which interferes with formation of such a polymerase chain reaction product when the primary oligonucleotide primers hybridize to any other nucleotide sequence present in the sample. The polymerase chain reaction products in a particular set may be distinguished from other polymerase chain reaction products with the same group or other groups. The primary oligonucleotide primers are blended with the sample and the polymerase to form a primary polymerase chain reaction mixture.

The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as described above. During the hybridization treatment, the target-specific portion of a primary oligonucleotide primer is hybridized to the target nucleotide sequences. In the extension treatment, the hybridized primary oligonucleotide primers are extended to form primary extension products complementary to the target nucleotide sequence to which the primary oligonucleotide primer is hybridized.

Although the upstream secondary primer-specific portions of a primary oligonucleotide primer set are not present on the target DNA, their sequences are copied by the second and subsequent cycles of the primary polymerase chain reaction phase. As a result, the primary extension products produced after the second and subsequent cycles have the secondary primer-specific portions on their 5' ends and the complement of primer-specific portion on their 3' ends.

In the second polymerase chain reaction phase of this aspect of the present invention, one or a plurality of secondary oligonucleotide primer sets are provided. Each set has a first secondary primer having a detectable reporter label and containing the same sequence as the 5' upstream portion of a first primary oligonucleotide primer, and a second secondary primer containing the same sequence as the 5' upstream primer of the second primary oligonucleotide primer from the same primary oligonucleotide primer set as the first primary oligonucleotide complementary to the first secondary primer. A set of secondary oligonucleotide primers amplify the primary extension products in a given group. The secondary oligonucleotide primers are blended with the primary extension products and the polymerase to form a secondary polymerase chain reaction mixture.

The secondary polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as described above. In the hybridization treatment, the secondary oligonucleotide primers are hybridized to the primary extension products, while the extension treatment causes the hybridized secondary oligonucleotide primers to be extended to form secondary extension products complementary to the primary extension products. After subjecting the secondary polymerase chain reaction mixture to the two or more polymerase chain reaction cycles, the labelled secondary extension products

are detected. This indicates the presence of one or more target nucleotide sequences in the sample.

The primary PCR/secondary PCR process of the present invention provides significant advantages over Southern hybridization, competitive PCR, and microsatellite marker analysis in detecting nucleotide deletions which cause a loss of heterozygosity. Although Southern hybridization is more accurate than competitive PCR, it is quite labor intensive, requires large amounts of DNA, and neither technique can be multiplexed. Current multiplex microsatellite marker approaches require careful attention to primer concentrations and amplification conditions.

The primary PCR/secondary PCR process of the present invention overcomes these difficulties encountered in the prior art. In the primary PCR phase, the primary oligonucleotide primers flank dinucleotide or other repeat sequences and include a secondary primer-specific portion. The primary PCR phase is carried out at low concentrations of these primers to allow several loci to undergo amplification at the same time. The secondary PCR phase causes amplification to continue at the same rate with target-specific secondary primers being selected to space one set of microsatellite markers from the adjacent set. The primary PCR/secondary PCR process can be used to carry out multiplex detection in a single PCR tube and with single gel lane analysis.

This aspect of the present invention is useful in carrying out a microsatellite marker analysis to identify nucleotide deletions in a gene. Such multiplex detection can be carried out in a single reaction tube. However, the primary PCR/secondary PCR process cannot distinguish amplifications from deletions, so, when making such distinctions, this process must be used in conjunction with the above-described LDR/PCR process or a differential PCR process.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a flow diagram depicting a primary PCR/secondary PCR/LDR process for detection of germline mutations, such as point mutations, by electrophoresis or capture on an addressable array. Note that the term "zipcode" which appears in FIG. **1** and other drawings refers to a sequence specific to a subsequently used primer or probe but not to either the target sequence or other genome sequences.

FIG. **2** is a flow diagram depicting a primary PCR/secondary PCR/LDR process for detection of biallelic polymorphisms by electrophoresis or capture on an addressable array.

FIG. **3** is a flow diagram depicting a primary PCR/secondary PCR/LDR process for detection of cancer-associated mutations by electrophoresis or capture on an addressable array.

FIG. **4** is a schematic diagram depicting a primary PCR/secondary PCR/LDR process for detection of biallelic polymorphisms.

FIG. **5** is a schematic diagram depicting a primary PCR/secondary PCR/LDR process for detection of allelic differences using LDR oligonucleotide probes which distinguish all possible bases at a given site.

FIG. **6** is a schematic diagram depicting a primary PCR/secondary PCR/LDR process for detection of the presence of any possible base at two nearby sites using LDR oligonucleotide probes which distinguish all possible bases at a given site.

FIG. **7** is a schematic diagram depicting a primary PCR/secondary PCR/LDR process for detection of cancer-associated mutations at adjacent alleles.

FIG. **8** is a flow diagram depicting an LDR/PCR process with and without restriction endonuclease digestion using electrophoresis detection.

FIG. **9** is a flow diagram depicting an LDR/PCR process using detection on an addressable array using gene-specific addresses.

FIG. **10** is a schematic diagram depicting an LDR/PCR process for multiplex detection of gene amplifications and deletions.

FIG. **11** is a schematic diagram depicting an allele specific problem for an LDR/PCR process.

FIG. **12** is a schematic diagram depicting a solution for the allele specific problem for an LDR/PCR process which is shown in FIG. **11**.

FIG. **13** is a flow diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of biallelic polymorphisms by electrophoresis or capture on an addressable array.

FIG. **14** is a flow diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of cancer-associated mutations by electrophoresis or capture on an addressable array.

FIG. **15** is a schematic diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of allele specific mutations and polymorphisms.

FIG. **16** is a schematic diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of mononucleotide repeat polymorphisms.

FIG. **17** is a schematic diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of mononucleotide repeat polymorphisms which are in low abundance.

FIG. **18** is a flow diagram depicting the detection of polymorphisms using an LDR/PCR process with an intermediate sequenase extension phase and a uracil N-glycosylase digestion phase after the LDR phase and before the PCR phase and with detection by electrophoresis or an addressable array.

FIG. **19** is a flow diagram depicting the detection of cancer using an LDR/PCR process with an intermediate sequenase extension phase and a uracil N-glycosylase digestion phase after the LDR phase and before the PCR phase and with detection by electrophoresis or an addressable array.

FIG. **20** is a schematic diagram depicting detection of mononucleotide repeats using an LDRIPCR process with an intermediate sequenase amplification phase and a uracil N-glycosylase digestion phase after the LDR phase and before the PCR phase.

FIG. **21** is a schematic diagram depicting detection of mononucleotide repeat polymorphisms which are in low abundance using an LDR/PCR process with an intermediate sequenase amplification phase and a uracil N-glycosylase digestion phase after the LDR phase and before the PCR phase.

FIG. **22** is a flow diagram depicting a primary PCR/secondary PCR process for detection of microsatellite repeats.

FIG. **23** is a schematic diagram depicting a primary PCR/secondary PCR process for multiplex detection of insertions and deletions in microsatellite repeats.

FIG. **24** shows the design of LDR oligonucleotide probes for quantification of gene amplifications and deletions in an LDR/PCR process.

FIGS. **25**A–D show electropherogram results for an LDR/PCR process.

FIGS. **26**A–C show electropherogram results for an LDR/PCR process of ErbB, G6PD, Int2, p53, and SOD gene segments from normal human female DNA and from DNA of the breast cancer cell line ZR-75-30 and the gastric carcinoma cell line SKGT-2. The ErbB gene is known to be amplified in the cancer cell lines. Target-specific ligation product sequences of 104 bp are generated in 10 cycles (94° C. for 30 sec, 65° C. for 4 min) of LDR using 500 femtomoles of each ligation primer, 50 ng of genomic DNA, 124 units of *Thermus thermophillus* ("Tth") ligase, 2 μl of 10X buffer (0.2 M Tris, pH 8.5 and 0.1 M $MgCl_2$), 2 μl of 10 mM NAD, and 1 μl of 200 mM DTT in a volume of 20 μl. The ligation products are proportionally amplified in 26 cycles (94° C. for 15 sec, 60° C. for 50 sec) of PCR by the addition of 30 μl of a solution containing 5 μl 10X Stoffel buffer (Perkin Elmer), 25 picomoles of each oligonucleotide primer, 2.5 units of Taq polymerase Stoffel fragment, and 8 μl of a solution 5 mM in each dNTP. After amplification, the products are digested with HaeIII and HinP1I to generate FAM-labeled products of 58 bp (ErbB (i.e. HER-2/neu/erbB oncogene)), 61 bp (G6PD), 67 bp (Int2 (i.e. int-2 oncogene)), 70 bp (p53) and 76 bp (SOD). These products are separated and analyzed on a 373A DNA sequencer using the Genescan 672 software package (Applied Biosystems, Inc., Foster City, Calif.). Results are displayed as electropherograms such that peak heights and areas reflect the amounts of the PCR products. In FIG. **26**A, the gene dosage determination for the five loci in normal human female DNA is shown. The peak heights and areas for G6PD, Int2, p53, and SOD are very similar. The peak height and area for ErbB is consistently small in normal genomic DNA. In FIG. **26**B, the peak height and area for ErbB are elevated when gene dosage is investigated in ZR-75-30, a cell line with known ErbB amplification. In FIG. **26**C, the gastric cell line, SKGT-2 shows dramatic amplification of the ErbB gene and a modest amplification of Int2. The G6PD gene peak may be embedded in the large ErbB peak.

FIGS. **27**A–C show electropherogram results for an LDR/PCR process to determine whether amplification of ErbB affected the relative peak heights of the other LDR oligonucleotide probes and PCR oligonucleotide primers for G6PD, Int2, p53, and SOD. In FIG. **27**A, the gene dosage determination for the four loci in normal human female DNA is shown. Peak heights and areas for G6PD, Int2, p53, and SOD are similar, as they were in the experiment using all five LDR primers. In FIG. **27**B, G6PD, Int2, and SOD analyzed in the ZR-75-30 breast cancer cell line show similar relative peak heights, comparable to their appearance in normal female DNA. The peak height for p53 is reduced, suggesting the deletion of this gene in a portion of the cells in this cell line. In FIG. **27**C, in the gastric carcinoma cell line, SKGT-2, G6PD, and p53 show comparable peak heights. The Int2 peak height remains relatively high, as it was in the experiment using all five LDR oligonucleotide probes. Thus, the LDR and PCR amplification of each product appears to be independent of the other products during the course of the experiment.

FIGS. **28**A–C show electropherogram results for the PCR phase of a primary PCR/secondary PCR/LDR process. Multiplex PCR amplification of 12 loci using primary PCR oligonucleotide primers produces approximately equal amounts of product. Over 80 gene regions with single-base polymorphisms were identified from the Human Genome Database. Twelve of these (see Table 10 and FIGS. **29**A–H) were amplified in a primary PCR phase as follows: Long primary PCR oligonucleotide primers were designed to have gene-specific 3' ends and 5' ends complementary to one of two sets of secondary PCR oligonucleotide primers. The upstream primary PCR oligonucleotide primers were synthesized with either FAM (i.e. 6-carboxyfluorescein; fluorescent dye used in sequencing and mutation detection) or TET (i.e. tetrachlorinated-6-carboxyfluorescein; fluorescent dye used in sequencing/mutation detection) fluorescent labels. All 24 base long primary PCR oligonucleotide primers were used at low concentration (2 picomole of each primer in 20 μl) in a 15 cycle primary PCR phase. After this, the two sets of secondary PCR oligonucleotide primers were added at higher concentrations (25 picomoles of each) and the secondary PCR phase was conducted for an additional 25 cycles. The products were separated on a 373 DNA Sequencer (Applied Biosystems). Panel A shows the electropherogram results for the FAM- and TET-labelled products combined. Panel B shows the FAM-labelled products alone. Panel C shows the TET-labelled products alone. The process produces similar amounts of multiplexed products without the need to adjust carefully primer concentrations or PCR conditions.

FIGS. **29**A–H show electropherogram results for the LDR phase of a primary PCR/secondary PCR/LDR process in detecting 12 biallelic genes for forensic identification. The primary and secondary PCR phases for the 12 polymorphic genes were performed as described in FIGS. **28**A–C. However, the secondary PCR oligonucleotide primers were not fluorescently labelled. The secondary PCR process extension products were diluted into a ligase buffer containing 36 LDR oligonucleotide probes (one common and two discriminating primers for each locus). LDR oligonucleotide probe sets were designed in two ways: (i) allele-specific oligonucleotide probes were of the same length but contained either the FAM or TET label; or (ii) the allele-specific oligonucleotide probes were both labelled with HEX (i.e. hexachlorinated-6-carboxyfluorescein; fluorescent dye used in sequencing and mutation detection) but differed in length by two basepairs. After 20 cycles of the LDR phase, the ligation product sequences were resolved using a 10% polyacrylamide sequencing gel on a 373 DNA Sequencer (Applied Biosystems). Panel A and E show the 12 loci PCR/LDR profiles of two individuals. Panels B, C, and D show, respectively, the FAM, TET, and HEX data for the individual in panel A. Panels F, G, and H show, respectively, the FAM, TET, and HEX data for the individual in panel E. The individual in panel A is homozygous only at locus 6 (ALDOB (i.e. aldolase B)) and locus 8 (IGF (i.e. insulin growth factor)). The individual in panel E is heterozygous only at loci 3 (C6 (i.e. complement component C6)), 5 (NF1 (i.e. neurofibromatosis)), 6 (ALDOB), and 8 (IGF). This demonstrates that the primary PCR/primary PCR/LDR process can simultaneously distinguish both homozygous and heterozygous genotypes at multiple positions.

DETAILED DESCRIPTION OF THE INVENTION

I. Primary PCR/Secondary PCR/LDR Process

One aspect of the present invention relates to a method for identifying two or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. This method involves a first polymerase chain reaction phase, a second polymerase chain reaction phase, and a ligase detection reaction phase. This process involves analyzing a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences.

In the first polymerase chain reaction phase, one or more primary oligonucleotide primer groups are provided. Each group comprises one or more primary oligonucleotide primer sets with each set having a first oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion, and a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion. The first oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion and the second oligonucleotide primers of each set in the same group contain the same 5' upstream primer-specific portion. The oligonucleotide primers in a particular set are suitable for hybridization on complementary strands of a corresponding target nucleotide sequence to permit formation of a polymerase chain reaction product. However, there is a mismatch which interferes with formation of such a polymerase chain reaction product when the primary oligonucleotide primers hybridize to any other nucleotide sequence present in the sample. The polymerase chain reaction products in a particular set may be distinguished from other polymerase chain reaction products in the same group or groups. The primary oligonucleotide primers, the sample, and the polymerase are blended to form a primary polymerase chain reaction mixture.

The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as substantially described above. During hybridization, target-specific portions of the primary oligonucleotide primers hybridize to the target nucleotide sequences. The extension treatment causes hybridized primary oligonucleotide primers to be extended to form primary extension products complementary to the target nucleotide sequence to which the primary oligonucleotide primers are hybridized.

Although the upstream secondary primer-specific portions of a primary oligonucleotide primer set are not present on the target DNA, their sequences are copied by the second and subsequent cycles of the primary polymerase chain reaction phase. As a result, the primary extension products produced after the second cycle have the secondary primer-specific portions on their 5' ends and the complement of the primer-specific portion on their 3' ends.

Next, there is a second polymerase chain reaction phase. This phase involves providing one or a plurality of secondary oligonucleotide primer sets. Each set has a first secondary oligonucleotide primer containing the same sequence as the 5' upstream portion of the first primary oligonucleotide primer, and a second secondary oligonucleotide primer containing the same sequence as the 5' upstream portion of the second primary oligonucleotide primer from the same primary oligonucleotide primer set as the first primary oligonucleotide complementary to the first secondary primer. A set of secondary oligonucleotide primers may be used to amplify all of the primary extension products in a given group. The secondary oligonucleotide primers are blended with the primary extension products and the polymerase to form a secondary polymerase chain reaction mixture.

The secondary polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles having a denaturation treatment, a hybridization treatment, and an extension treatment, as substantially set forth above. During the hybridization treatment, the secondary oligonucleotide primers hybridize to complementary sequences present on the primary extension products but not to the original target sequence. The extension treatment causes the hybridized secondary oligonucleotide primers to be extended to form secondary extension products complementary to the primary extension products.

The last phase of this aspect of the present invention involves a ligase detection reaction process. Here, a plurality of oligonucleotide probe sets are provided where each set has a first oligonucleotide probe, having a secondary extension product-specific portion and a detectable reporter label, and a second oligonucleotide probe, having a secondary extension product-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a complementary secondary extension product-specific portion. However, there is a mismatch which interferes with such ligation when the oligonucleotide probes are hybridized to any other nucleotide sequence present in the sample. The ligation product of oligonucleotide probes in a particular set may be distinguished from either individual probes or other ligation products. The plurality of oligonucleotide probe sets, the secondary extension products, and a ligase are blended to form a ligase detection reaction mixture.

The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles having a denaturation treatment and hybridization treatment substantially as described above. In the hybridization treatment, the oligonucleotide probe sets hybridize at adjacent positions in a base-specific manner to the respective secondary extension products if present. As a result, adjacent probes ligate to one another to form a ligation product sequence containing the detectable reporter label and the secondary extension product-specific portions connected together. The oligonucleotide probe sets may hybridize to nucleotide sequences other than the respective complementary secondary extension products but do not ligate together due a presence of one or more mismatches and individually separate during the denaturation treatment. Following the ligation detection reaction cycles, the reporter labels oft he ligation product sequences are detected which indicates the presence of one or more target nucleotide sequences in the sample.

FIGS. **1**, **2**, and **3** show flow diagrams of the primary PCR/secondary PCR/LDR process of the present invention utilizing either of two detection procedures. One alternative involves use of capillary electrophoresis or gel electrophoresis and a fluorescent quantification procedure. Alternatively, detection can be carried out by capture on an array of capture oligonucleotide addresses and fluorescent quantification. FIG. **1** relates to detection of germline mutations (e.g., a point mutation), while FIG. **2** detects biallelic polymorphisms, and FIG. **3** shows the detection of cancer-associated mutations.

FIG. **1** depicts the detection of a germline point mutation. In step 1, after DNA sample preparation, multiple exons are subjected to primary PCR amplification using Taq (i.e. *Thermus aquaticus*) polymerase under hot start conditions with oligonucleotide primers having a target-specific portion and a secondary primer-specific portion. At the end of the primary PCR phase, Taq polymerase may be inactivated by heating at 100° C. for 10 min or by a freeze/thaw step. The products of the primary PCR amplification phase are then subjected in step 2 to secondary PCR amplification using Taq polymerase under hot start conditions with the secondary oligonucleotide primers. At the end of the secondary PCR phase, Taq polymerase may be inactivated by heating at 100° C. for 10 min or by a freeze/thaw step. In step 3, products of the secondary PCR phase are then diluted 20-fold into fresh LDR buffer containing LDR oligonucle-

otide probes containing allele-specific portions and common portions. Step 4 involves the LDR phase of the process which is initiated by addition of Taq ligase under hot start conditions. During LDR, oligonucleotide probes ligate to their adjacent oligonucleotide probes only in the presence of target sequence which gives perfect complementarity at the junction site. The products may be detected in two different formats. In the first format 5a, fluorescently-labeled LDR probes contain different length poly A or hexaethylene oxide tails. Thus, each ligation product sequence (resulting from ligation of two probes hybridized on normal DNA) will have a slightly different length and mobility such that several ligation product sequences yield a ladder of peaks. Thus, each ligation product (resulting from ligation of two probes hybridized on normal DNA) will have a slightly different length and mobility such that several ligation product sequences yield a ladder of peaks. A germline mutation would generate a new peak on the electropherogram. Alternatively, the LDR probes may be designed such that the germline mutation ligation product sequence migrates with the same mobility as a normal DNA ligation product sequence, but it is distinguished by a different fluorescent reporter. The size of the new peak will approximate the amount of the mutation present in the original sample; 0% for homozygous normal, 50% for heterozygous carrier, or 100% for homozygous mutant. In the second format 5b, each allele-specific probe contains e.g., 24 additional nucleotide bases on their 5' ends. These sequences are unique addressable sequences which will specifically hybridize to their complementary address sequences on an addressable array. In the LDR reaction, each allele-specific probe can ligate to its adjacent fluorescently labeled common probe in the presence of the corresponding target sequence. Ligation product sequences corresponding to wild type and mutant alleles are captured on adjacent addresses on the array. Unreacted probes are washed away. The black dots indicate 100% signal for the wild type allele. The white dots indicate 0% signal for the mutant alleles. The shaded dots indicate the one position of germline mutation, 50% signal for each allele.

FIG. **2** depicts the detection of biallelic polymorphisms. In step 1, after DNA sample preparation, multiple exons are subjected to primary PCR amplification using Taq polymerase under hot start conditions with oligonucleotide primers having a target-specific portion and a secondary primer-specific portion. The products of the primary PCR amplification phase are then subjected in step 2 to secondary PCR amplification using Taq polymerase under hot start conditions with the secondary oligonucleotide primers. At the end of the secondary PCR phase, Taq polymerase may be inactivated by heating at 100° C. for 10 min or by a freeze/thaw step. In step 3, products of the secondary PCR phase are then diluted 20-fold into fresh LDR buffer containing LDR oligonucleotide probes containing allele-specific portions and common portions. Step 4 involves the LDR phase of the process which is initiated by addition of Taq ligase under hot start conditions. During LDR, oligonucleotide probes ligate to their adjacent oligonucleotide probes only in the presence of target sequence which gives perfect complementarity at the junction site. In the first format 5a, fluorescently-labeled LDR probes contain different length poly A or hexaethylene oxide tails. Each ligation product sequence will have a slightly different length and mobility, such that several LDR products yield a ladder of peaks. Alternatively, the LDR probes may be designed such that the ligation products for polymorphic alleles migrate at the same position but are distinguished by different fluorescent reporter groups. The size of the peaks will approximate the amount of each allele. In the second format Sb, each oligonucleotide probe contains unique addressable sequences with e.g., 24 additional nucleotide bases on their 5' ends. These sequences will specifically hybridize to their complementary address sequences on an array of capture oligonucleotides. In the LDR phase, each allele-specific probe can ligate to its adjacent fluorescently labeled common probe in the presence of corresponding target sequence. Ligation product sequences corresponding to each allele are captured on the array, while unligated oligonucleotide probes are washed away. The black dots indicate that both chromosomes have a given allele, the white dots show that neither chromosome has that allele, and the shaded dots indicate that one chromosome has a given allele.

FIG. **3** depicts the detection of cancer-associated mutation. In step 1, after DNA sample preparation, multiple exons are subjected to primary PCR amplification using Taq polymerase under hot start conditions with oligonucleotide primers having a target-specific portion and a secondary primer-specific portion. The products of the primary PCR amplification phase are then subjected in step 2 to secondary PCR amplification using Taq polymerase under hot start conditions with the secondary oligonucleotide primers. At the end of the secondary PCR phase, Taq polymerase may be inactivated by heating at 100° C. for 10 min or by a freeze/thaw step. Fluorescent quantification of PCR products can be achieved using capillary or gel electrophoresis in step 3. In step 4, the products are spiked with a 1/100 dilution of marker DNA (for each of the fragments). This DNA is homologous to wild type DNA, except it contains a mutation which is not observed in cancer cells, but which may be readily detected with the appropriate LDR probes. In step 5, the mixed DNA products in products of the secondary PCR phase are then diluted 20-fold into fresh LDR buffer containing LDR oligonucleotide probes containing allele-specific portions and common portions. Step 6 involves the LDR phase of the process which is initiated by addition of Taq ligase under hot start conditions. During LDR, oligonucleotide probes ligate to their adjacent oligonucleotide probes only in the presence of target sequence which gives perfect complementarity at the junction site.

The products may be detected in the same two formats discussed above. In the format of step 7a, products are separated by capillary or gel electrophoresis, and fluorescent signals are quantified. Ratios of mutant peaks to marker peaks give the approximate amount of cancer mutations present in the original sample divided by 100. In the format of step 7b, products are detected by specific hybridization to complementary sequences on an addressable array. Ratios of fluorescent signals in mutant dots to marker dots give the approximate amount of cancer mutations present in the original sample divided by 100.

As shown in FIG. **4**, two DNA fragments of interest are treated with the primary PCR/secondary PCR/LDR process of the present invention. Initially, the double stranded DNA molecules are denatured to separate the strands. This is achieved by heating to a temperature of 80–105° C. Low concentrations of primary PCR oligonucleotide primers, containing a 3' target-specific portion (shaded area) and 5' secondary primer-specific portion (black area), are then added and allowed to hybridize to the strands, typically at a temperature of 50–85° C. A thermostable polymerase (e.g., Taq aquaticus polymerase) is also added, and the temperature is then adjusted to 50–85° C. to extend the primer along the length of the nucleic acid to which the primer is hybridized. After the extension phase of the polymerase

chain reaction, the resulting double stranded molecule is heated to a temperature of 80–105° C. to denature the molecule and to separate the strands. These hybridization, extension, and denaturation steps may be repeated a number of times to amplify the target to an appropriate level.

In the secondary PCR phase, the products of the primary PCR phase are blended with secondary PCR oligonucleotide primers and allowed to hybridize to one another, typically at a temperature of 50–85° C. The secondary oligonucleotide primers are usually used in higher concentrations than are the primary oligonucleotide primers. Taq polymerase is also added, and the temperature is then adjusted to 50–85° C. to extend the primer along the length of the primary PCR extension products to which the secondary oligonucleotide primer is hybridized. After the extension phase of the polymerase chain reaction, the resulting double stranded molecule is heated to a temperature of 80–105° C. to denature the molecule and to separate the strands. These hybridization, extension, and denaturation steps may be repeated a number of times to amplify the target to an appropriate level.

Once the secondary PCR phase of the process is completed, the ligation detection reaction phase begins, as shown in FIG. **4**. After denaturation of the target nucleic acid, if present as a double stranded DNA molecule, at a temperature of 80–105° C., preferably 94° C., ligation detection reaction oligonucleotide probes for one strand of the target nucleotide sequence are added along with a ligase (for example, as shown in FIG. **4**, a thermostable ligase like *Thermus aquaticus* ligase). The oligonucleotide probes are then allowed to hybridize to the target nucleic acid molecule and ligate together, typically, at a temperature of 45–85° C., preferably, 65° C. When there is perfect complementarity at the ligation junction, the oligonucleotides can be ligated together. Where the variable nucleotide is T or A, the presence of T in the target nucleotide sequence will cause the oligonucleotide probe with the F1 reporter label to ligate to the common oligonucleotide probe with the 5' poly A tail A_n, and the presence of A in the target nucleotide sequence will cause the oligonucleotide probe with the F2 reporter label to ligate to the common oligonucleotide probe with A_n. Similarly, where the variable nucleotide is A or G, the presence of T in the target nucleotide sequence will cause the oligonucleotide probe with F3AA reporter label (i.e. the F3 reporter label coupled to 2 additional bases forming a 5' poly A spacer) to ligate to the common oligonucleotide probe with the 5' poly A tail A_{n+4}, and the presence of C in the target nucleotide sequence will cause the oligonucleotide probe with the F3 reporter label to ligate to the common oligonucleotide probe with the 5' poly A tail A_{n+4}. Following ligation, the material is again subjected to denaturation to separate the hybridized strands. The hybridization/ligation and denaturation steps can be carried out through one or more cycles (e.g., 1 to 50 cycles) to amplify target signals. Equimolar ligation of both F3-labeled oligonucleotides indicates the individual is heterozygous for that locus, whereas ligation of only the F2 labeled oligonucleotides indicates the individual is homozygous for the other locus.

In FIG. **4**, the poly A_n and poly A_{n+4} tails are used where quantification is to be carried out by capillary or gel electrophoresis. The tails of differing length cause the corresponding different ligation product sequences to form bands at different locations in the gel or capillary. The presence of these bands at different locations permit the corresponding nucleotide differences in the DNA being analyzed to be identified. Although ligation product sequences can be distinguished based on the use of different reporter labels, the combination of different reporter labels and different length tails permits greater numbers of nucleotide differences to be distinguished. This is important for multiplex detection processes.

As noted above with respect to FIGS. **1** to **3**, detection can be carried out on an addressable array instead of with gel or capillary electrophoresis. The use of such addressable arrays require that the poly A tails on the LDR oligonucleotide probe not containing a reporter label (i.e. tails A_n and A_{n+4}) be replaced with different addressable array-specific oligonucleotide portions. As explained more fully infra, a solid support is provided with an array of capture oligonucleotides, some of which are complementary to the different addressable array-specific oligonucleotide portions. Hybridization of these portions to their complementary capture oligonucleotide probes indicates the presence of a corresponding nucleotide difference.

FIG. **5** is a schematic diagram of a primary PCR/secondary PCR/LDR process, in accordance with the present invention, where any possible base in 2 DNA molecules of interest are distinguished. The primary and secondary PCR processes are carried out in substantially the same way as described for FIG. **4**. Appearance of fluorescent reporter labels F1, F2, F3, and F4 in conjunction with the left hand DNA molecule indicates the presence of the A, G, C, and T alleles in the DNA molecule, respectively. As shown in FIG. **5**, equal amounts of the F1 and F3 reporter labels indicates that the individual in question is heterozygous for the A and C alleles. With respect to analysis of the right hand DNA molecule in FIG. **5**, the same reporter label is used to indicate the presence of the different alleles; however, on each oligonucleotide probe with the distinguishing bases, there are different 5' poly A tails. More particularly, a 2 unit poly A tail, a 4 unit poly A tail, a 6 unit poly A tail, and an 8 unit poly A tail correspond to the T, C, G, and A alleles in the DNA molecule, respectively. As shown in FIG. **5**, equal amounts of the F1 reporter label with the A6 and A4 tails indicates that the individual in question is heterozygous for the G and C alleles.

FIG. **6** is a schematic diagram of a primary PCR/secondary PCR/LDR process, in accordance with the present invention, for detecting the presence of any possible base at two nearby sites in DNA molecules of interest. The primary and secondary PCR phases are carried out in substantially the same way as described for FIG. **4**. Here, the LDR probes are able to overlap, yet are still capable of ligating provided there is perfect complementarity at the junction. This distinguishes LDR from other approaches, such as allele-specific PCR where overlapping primers would interfere with one another. In FIG. **6**, the discriminating oligonucleotide probes contain the reporter label with the discriminating base on the 3' end of these probes. The poly A tails are on the 3' end of common oligonucleotide probes. In the left hand DNA molecule, the presence of equal amounts of ligation product sequences with reporter labels F1 and F3 shows that the individual in question is heterozygous for the A and C alleles in the first position. Similarly, in the second position for the left hand DNA molecule, the presence of ligation product sequences with reporter labels F2, F3, and F4 shows that the individual in question is heterozygous for the G, C, and T alleles. Turning to the right hand DNA molecule, the presence of equal amounts of ligation product sequences with reporter label F1 having the A_6 and A_4 tails indicates that at the first position, the individual in question is heterozygous for the G and C alleles. In the second position for the right hand DNA molecule, the presence of the equal amounts of ligation product sequences with

reporter label F1 having the A_8 and A_2 tails indicates that the individual in question is heterozygous for the A and T alleles.

FIG. **7** is a schematic diagram depicting the use of the primary PCR/secondary PCR/LDR process of the present invention to detect a low abundance mutation in the presence of an excess of normal sequence. Here, in the left hand DNA molecule is codon 12 of the K-ras gene, sequence GGT, which codes for glycine ("Gly"). A small percentage of the cells contain the G to A mutation in GAT, which codes for aspartic acid ("Asp"). The LDR probes for wild-type (i.e. normal) sequences are missing from the reaction. If the normal LDR probes (with the discriminating base being G) were included, they would ligate to the common probes and overwhelm any signal coming from the mutant target. Instead, as shown in FIG. **7**, the existence of a ligation product sequence with fluorescent label F1 and the A_{n+2} tail indicates the presence of the aspartic acid encoding mutant. In the right hand DNA molecule, FIG. **7** shows codon 61 of the K-ras gene sequence CAG which codes for glutamine ("Gln"). A small percentage of the cells contain the C to G mutation in GAG, which codes for glutamic acid ("Glu"). Again, the LDR oligonucleotide probes do not include the C and A bases found in the wild type form to avoid overwhelming the mutant signal. For this DNA molecule, the existence of a ligation product sequence with fluorescent label F2 and the A_{n+4} tail indicates the presence of the glutamic acid encoding mutant.

II. LDR/PCR Process

A second aspect of the present invention relates to a method for identifying one or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. This method has a ligase detection reaction phase followed by a polymerase chain reaction phase. This method involves providing a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences.

In the ligase detection reaction phase, one or more of oligonucleotide probe sets are provided. Each set has a first oligonucleotide probe, having a target-specific portion and a 5' upstream primer-specific portion, and a second oligonucleotide probe, having a target-specific portion and a 3' downstream primer-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target nucleotide sequence. However, there is a mismatch which interferes with such ligation when they are hybridized to any other nucleotide sequence present in the sample. The sample, the plurality of oligonucleotide probe sets, and a ligase are blended together to form a ligase detection reaction mixture.

The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles. These cycles include a denaturation treatment and a hybridization treatment. In the denaturation treatment, any hybridized oligonucleotides are separated from the target nucleotide sequences. The hybridization treatment causes the oligonucleotide probe sets to hybridize at adjacent positions in a base-specific manner to their respective target nucleotide sequences if present in the sample. Once hybridized, the oligonucleotide probe sets ligate to one another to form a ligation product sequence. This product contains the 5' upstream primer-specific portion, the target-specific portions connected together, and the 3' downstream primer-specific portion. The ligation product sequence for each set is distinguishable from other nucleic acids in the ligase detection reaction mixture. The oligonucleotide probe sets hybridized to nucleotide sequences in the sample other than their respective target nucleotide sequences but do not ligate together due to a presence of one or more mismatches and individually separate during the denaturation treatment.

In the polymerase chain reaction, one or a plurality of oligonucleotide primer sets are provided. Each set has an upstream primer containing the same sequence as the 5' upstream primer-specific portion of the ligation product sequence and a downstream primer complementary to the 3' downstream primer-specific portion of the ligation product sequence, where one primer has a detectable reporter label. The ligase detection reaction mixture is blended with the one or a plurality of oligonucleotide primer sets and the polymerase to form a polymerase chain reaction mixture.

The polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles which include a denaturation treatment, a hybridization treatment, and an extension treatment. During the denaturation treatment, hybridized nucleic acid sequences are separated. The hybridization treatment causes primers to hybridize to their complementary primer-specific portions of the ligation product sequence. During the extension treatment, hybridized primers are extended to form extension products complementary to the sequences to which the primers are hybridized. In a first cycle of the polymerase chain reaction phase, the downstream primer hybridizes to the 3' downstream primer-specific portion of the ligation product sequence and is extended to form an extension product complementary to the ligation product sequence. In subsequent cycles, the upstream primer hybridizes to the 5' upstream primer-specific portion of the extension product complementary to the ligation product sequence and the 3' downstream primer hybridizes to the 3' downstream portion of the ligation product sequence.

Following the polymerase chain reaction phase of this process, the reporter labels are detected and the extension products are distinguished to indicate the presence of one or more target nucleotide sequences in the sample.

FIG. **8** is a flow diagram depicting the LDR/PCR process of the present invention with or without restriction endonuclease digestion and using capillary electrophoresis detection. In step 1, a DNA sample is mixed with Taq ligase and oligonucleotide probes containing a target-specific portion and a primer-specific portion. The mixture is subjected to an LDR process to produce ligation product sequences containing the ligated target-specific portions and the primer-specific portions. Step 2 involves mixing the ligation product sequences with Taq polymerase and primers and subjecting the mixture to a PCR process. The next step is determined as a function of whether the ligation product sequences are the same or different sizes. Where the ligation product sequences are different sizes, step 3a is selected which involves subjecting the extension products from PCR to capillary electrophoresis or gel electrophoresis, either of which is followed by fluorescent quantification. Step 3b is utilized where the ligation product sequences are the same size and involves subjecting the extension products from the PCR phase to restriction endonuclease digestion. This generates digestion fragments of unique size which can be subjected to capillary electrophoresis or gel electrophoresis, followed by fluorescent quantification, according to step 4b. When step 3a is selected, the curve generated as a result of electrophoresis shows three ligation product sequences migrating at lengths of 104, 107, and 110, with the peak areas representing amplification of the Her-2 gene, loss of

heterozygosity of the p53 gene, and the control SOD gene, respectively. The electrophoresis curve where steps 3b and 4b are used involves three ligation product sequence restriction fragments at lengths of 58, 70, and 76, with the peak areas representing amplification of the Her-2 gene, loss of heterozygosity of the p53 gene, and the control SOD gene, respectively.

As an alternative to FIG. **8**, FIG. **9** shows the LDR/PCR process of the present invention where, in step 3, the extension products are captured on an array of capture oligonucleotide addresses. The capture oligonucleotide probes can be complementary to a nucleotide sequence across the ligation junction. The number of gene copies captured on the array of capture oligonucleotides is then determined by fluorescent quantification as compared with known controls. In FIG. **8**, such analysis of the array indicates ligation product sequences hybridizing to gene-specific addresses, where the fluorescent intensity represents amplification of the Her-2 gene, loss of heterozygosity of the p53 gene, and the control SOD gene, respectively.

FIG. **10** is a schematic diagram depicting an LDR/PCR process for multiplex detection of gene amplifications and deletions. Here, the ratio of the Her-2/neu gene from Chromosome 17q, the p53 gene from Chromosome 17p, and the SOD gene from Chromosome 21q is detected. Following denaturation of DNA at 94° C., pairs of oligonucleotide probes, having a target-specific portion and a primer-specific portion, are allowed to anneal adjacent to each other on target nucleic acids and ligate to one another (in the absence of mismatches). This ligase detection reaction is carried out with Tth ligase at a hybridization/ligation temperature of 65° C. which is well below the T_m values of 75° C. for the oligonucleotide probes. Next, the ligation product sequences are simultaneously amplified by PCR using Taq polymerase and two common primers complementary to the primer-specific portion, one of which is fluorescently labeled. This maintains the proportionality of the target sequences initially present in the sample. The extension products are then digested with HaeIII and Hinp1I which releases fluorescently labeled fragments of unique sizes for each target sequence present in the sample. The digestion products are separated and analyzed on an Applied Biosystems, Inc. (Foster City, Calif.) 373A DNA Sequencer. The peak heights and areas are related to the relative copies of genes present in the initial target sample.

FIG. **11** is a schematic diagram, depicting a problem which can be encountered with the allele-specific LDR/PCR process. While a PCR/LDR process is very powerful, there may be circumstances where a multiplexed allele-specific LDR/PCR process of the present invention would be preferred. The concept is to have one or more sets of LDR oligonucleotide probes, each set characterized by (a) a first oligonucleotide probe, having a target-specific portion and a 5' upstream primer-specific portion, and (b) a second oligonucleotide probe, having a target-specific portion and a 3' downstream primer-specific portion. As shown in step 1 of FIG. **11**, the LDR oligonucleotide probes anneal adjacent to each other on the target sequence. An LDR reaction using thermostable ligase (black dot) would form a ligation product sequence provided there is perfect complementarity at the ligation junction. In step 2, the ligation product sequences are PCR amplified with primer sets, each set characterized by (a) an upstream primer containing the same sequence as the 5' upstream primer-specific portion of a ligation product sequence and (b) a downstream primer complementary to the 3' downstream primer-specific portion of that ligation product sequence. The primers are shown as black lines in step 2. If one primer is fluorescently labeled, it will generate a fluorescent product which may be detected in a variety of detection schemes. For the LDR/PCR process to be specific, a PCR extension product should not be formed in the absence of a ligation event. Unfortunately, the possibility exists for polymerase to extend the first LDR oligonucleotide probe (off normal target), forming a product containing the length of the target sequence, and a primer-specific portion on the 5' end. Meanwhile, polymerase can make several complementary copies of the downstream LDR probe using the downstream primer. In a second amplification cycle, this downstream LDR probe extension product can anneal to the upstream LDR probe extension product off the target sequence, and generate a sequence containing the target region flanked by the two primer-specific sequences. This product will amplify as the LDR product, and thus yield a false positive signal.

FIG. **12** is a schematic drawing showing a solution to the allele specific LDR/PCR problem, utilizing an intermediate exonuclease digestion step. Allele-specific LDR/PCR can be achieved while significantly reducing background ligation independent (incorrect) target amplification. To do so, it is necessary to eliminate one or more of the components required for ligation independent PCR amplification, without removing the information content of the ligation product sequence. One solution is to use exonuclease in step 2 to digest unreacted LDR oligonucleotide probes from step 1. By blocking the end which is not ligated, for example the 3' end of the downstream oligonucleotide probe, one probe can be made substantially resistant to digestion, while the other is sensitive. Only the presence of full length ligation product sequence will prevent digestion of the upstream primer. Blocking groups include use of a thiophosphate group and/or use of 2-O-methyl ribose sugar groups in the backbone. Exonucleases include Exo I (3'–5'), Exo III (3'–5'), and Exo IV (both 5'–3' and 3'–5'), the later requiring blocking on both sides. One convenient way to block both probes is by using one long "padlock" probe (see M. Nilsson et. al., "Padlock Probes: Circularizing Oligonucleotides for Localized DNA Detection," *Science* 265: 2085–88 (1994), which is hereby incorporated by reference), although this is by no means required. An advantage of using exonucleases, for example a combination of Exo I (single strand specific) and Exo III (double strand specific), is the ability to destroy both target and one LDR probe, while leaving the ligation product sequences substantially undigested. By using an exonuclease treatment prior to PCR, in accordance with steps 3 and 4, either one or both oligonucleotide probes in each set are substantially reduced, and thus hybridization of the remaining oligonucleotide probes to the original target DNA (which is also substantially reduced by exonuclease treatment) and formation of a ligation product sequence which is a suitable substrate for PCR amplification by the oligonucleotide primer set is substantially reduced. In other words, formation of ligation independent labeled extension products is substantially reduced or eliminated.

FIG. **13** is a flow diagram showing an allele-specific LDR/PCR process using exonuclease digestion with either size based- or DNA array based-detection. The flow diagram shows the three reactions required for the multiplexed allele-specific LDR/PCR process. In step 1, sets of LDR oligonucleotide probes (wherein the downstream probes are blocked on their 3' ends) are ligated in the presence of the correct allele target using Taq DNA ligase. Unreacted upstream probes are digested with exonuclease in step 2, coincidentally, target is also digested. Finally, in step 3, primer sets are used to amplify the ligation product sequence

by hybridizing to the primer specific portions of ligation product sequences. In step 4a, the LDR oligonucleotide probes in a given particular set generate a unique length product, and thus may be distinguished from either oligonucleotide probes or other ligation products. After the PCR reaction, the products are separated by size or electrophoretic mobility. Labels on the PCR primers are detected, and the products are distinguished by size. In step 4b, the LDR oligonucleotide probes in a particular set use may be distinguished from either oligonucleotide probes or other ligation product sequences by differences in the sequences of the PCR primers. By using a plurality of oligonucleotide primer sets, each set characterized by (a) an upstream primer containing the same sequence as the 5' upstream primer-specific portion of a ligation product sequence, and (b) a downstream primer complementary to the 3' downstream primer-specific portion of that ligation product sequence, wherein one primer has a detectable reporter label and the other primer contains an addressable nucleotide sequence linked to the 5' end of that primer such that the addressable nucleotide sequence remains single stranded after a PCR reaction, all the products can be distinguished. The latter may be achieved by using a non-natural base within a PCR primer which polymerase cannot extend through, thus generating PCR products which have single stranded tails. See C. Newton, et. al., "The Production of PCR Products with 5' Single-stranded Tails Using Primers that Incorporate Novel Phosphoramidite Intermediates," *Nucl. Acids Res.* 21(3): 1155–62 (1993), which is hereby incorporated by reference. By providing a DNA array with different capture oligonucleotides immobilized at different particular sites, where the capture oligonucleotides have nucleotide sequences complementary to the addressable nucleotide sequences on the primers, the PCR extension products can hybridize to the DNA array. Finally, the labels of extension product sequences captured using the addressable array-specific portions immobilized to the DNA array at particular sites can be detected. This indicates the presence of one or more target nucleotide sequences in the sample.

FIG. **14** is a flow diagram showing a quantitative allele-specific LDR/PCR process using exonuclease digestion in step 3 with either size based- or DNA array based-detection. The flow diagram shows how one can quantify the amounts of different targets (especially low abundance cancer mutations) by adding marker sequence(s) (step 1) at the start of the LDR reaction (step 2). In this embodiment, the biochemical reactions (i.e. PCR (step 4)) are followed, as described in FIG. **13**, and the relative amount of mutant product to marker product are quantified using capillary or gel electrophoresis (step 5a) or capture on an addressable array (step 5b). The amount of mutant target present in the original sample can then be determined.

FIG. **15** is a schematic drawing showing an allele-specific LDR/PCR process with exonuclease digestion (step 2) for detection of mutations or polymorphisms. Mutations and polymorphisms may be distinguished as described in FIG. **12**. In this example, in step 1, the upstream LDR oligonucleotide probes, which have the discriminating allele-specific base at the 3' end of the target-specific portion, have different 5' upstream primer-specific portions. Thus, different primers (in the PCR amplification step (i.e. step 3)) may be labeled with different fluorescent groups (Fam and Tet) to allow for distinction of products (step 4). An array based detection scheme may also be used, where the upstream (allele-specific) probes have different 5' upstream primer-specific portions, and the different PCR primers contain different addressable nucleotide sequences which remain single stranded after a PCR reaction.

FIG. **16** is a schematic drawing showing an allele-specific LDR (step 1)/PCR (step 3) process using exonuclease digestion (step 2) for detection of mononucleotide or dinucleotide repeat polymorphisms. One of the most powerful uses of LDR/PCR is for detecting nucleotide repeat polymorphisms, a task which cannot be achieved by allele-specific PCR (because the 3' nucleotide is always the same), nor easily achieved by observing PCR product size variation (due to Taq polymerase slippage during amplification). In FIG. **16**, the LDR oligonucleotide probes distinguish between an A_9 and A_{10} mononucleotide repeat sequence by virtue of the specificity of thermostable DNA ligase. LDR products are only formed on the correct length target sequence, and thus the presence of that target is distinguished (step 4).

FIG. **17** is schematic drawing showing an allele-specific LDR/PCR process using exonuclease digestion (step 2) for detection of low abundance mononucleotide or dinucleotide repeat mutations. Mononucleotide repeat length mutations may be distinguished as described in FIG. **12**. In FIG. **17**, the LDR oligonucleotide probes (step 1) distinguish between an A_8, A_9 (mutants), and A_{10} (normal) mononucleotide repeat sequences by virtue of the specificity of thermostable DNA ligase. The two upstream LDR oligonucleotide probes differ in the length of the mononucleotide sequence at their 3' ends of their target specific portion and have different 5' upstream primer-specific portions. Thus, different primers (in the PCR amplification step (step 3)) may be labeled with different fluorescent groups (Fam and Tet) to allow for distinction of products (step 4). This has the distinct advantage of allowing mononucleotide repeat polymorphisms to be distinguished based on fluorescent label instead of size, the latter being susceptible to false positives due to polymerase slippage. An array based detection scheme may also be used, where the upstream (allele-specific) probes have different 5' upstream primer-specific portions, and the different PCR primers contain different addressable nucleotide sequences which remain single stranded after a PCR reaction.

FIG. **18** is a flow diagram, showing an allele-specific LDR/PCR process using uracil N-glycosylase selection with either size based- or DNA array based-detection. The flow diagram shows the four reactions required for multiplexed allele-specific LDR/PCR. Sets of LDR oligonucleotide probes (wherein one or both probes contain deoxy-uracil in place of deoxythimidine) are ligated in the presence of the correct allele target using Tdq DNA ligase in step 1. A complementary copy of the ligation product sequence is made with sequenase in step 2. Sequenase is a modified T7 polymerase, with any easily inactivated polymerase (i.e. mesophilic polymerases such as, *E. coli* polymerase) being useful. Both ligation product sequences and unreacted probes are destroyed with uracil N-glycosylase in step 3. The advantage of using uracil N-glycosylase is its proven ability in carry-over prevention for PCR. Finally PCR primer sets are used to amplify the sequenase extension products in step 4. In step 5a, the LDR oligonucleotide probes in a particular set generate a unique length product, and thus may be distinguished from either probes or other ligation products. After the PCR reaction, the products are separated by size or electrophoretic mobility. Labels on the PCR primers are detected, and products are distinguished by size. In step 5b, the LDR oligonucleotide probes in a particular set may be distinguished from either probes or other ligation product sequences by differences in the sequences of the primer-specific portions. By using a plu-

rality of oligonucleotide primer sets, each set characterized by (a) an upstream primer containing the same sequence as the 5' upstream primer-specific portion of a ligation product sequence, and (b) a downstream primer complementary to the 3' downstream primer-specific portion of that ligation product sequence. One primer has a detectable reporter label, and the other primer contains an addressable array-specific portion linked to the 5' end of that primer such that the addressable array-specific portion remains single stranded after a PCR reaction, one can distinguish all the products. By providing a DNA array with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the addressable array-specific portions on the primers, the PCR extension products can be hybridized to the DNA array. Finally, the labels of extension product sequences captured using the addressable nucleotide sequence portions and immobilized to the DNA array at particular sites can be detected to indicate the presence of one or more target nucleotide sequences in the sample.

FIG. **19** is a flow diagram showing a quantitative allele-specific LDR/PCR process using uracil N-glycosylase selection with either size based- or DNA array based-detection. The flow diagram shows how one can quantify the amounts of different targets (especially low abundance cancer mutations) by adding marker sequence(s) in step 1 at the start of the LDR phase in step 2. As described in FIG. **18**, the biochemical reactions (i.e. sequenase treatment (step 3), uracil N-glycosylase selection (step 4), and PCR (step 5)) are preceded with, and the relative amount of mutant product to marker product is quantified using capillary or gel electrophoresis (step 6a) or capture on an addressable array (step 6b). From this information, the amount of mutant target present in the original sample can be determined.

FIG. **20** is a schematic drawing showing an allele-specific LDRIPCR process using uracil N-glycosylase selection (step 3) (after sequenase treatment (step 2)) for detection of mononucleotide or dinucleotide repeat polymorphisms. One of the most powerful uses of the LDR/PCR process is for detecting nucleotide repeat polymorphisms, a task which cannot be achieved by allele-specific PCR (since the 3' nucleotide is always the same), nor easily achieved by observing PCR product size variation (due to Taq polymerase slippage during amplification), as in step 4. In FIG. **20**, the LDR (step 1) oligonucleotide probes distinguish between an A_9 and A_{10} mononucleotide repeat sequence by virtue of the specificity of thermostable DNA ligase. Ligation product sequences are only formed on the correct length target sequence, and, thus, the presence of that target is distinguished in step 5.

FIG. **21** is a schematic drawing showing an allele-specific LDR/PCR process using uracil N-glycosylase selection for detection of low abundance mononucleotide or dinucleotide repeat mutations. Mononucleotide repeat length mutations may be distinguished as described in FIG. **18**. In FIG. **21**, the LDR oligonucleotide probes distinguish between an A_8, A_9 (mutants), and A_{10} (normal) mononucleotide repeat sequences by virtue of the specificity of thermostable DNA ligase (step 1). Sequenase treatment (step 2) and uracil N-glycosylase selection (step 3) are then carried out. The two upstream LDR oligonucleotide probes differ in the length of the mononucleotide sequence at the 3' ends of their target-specific portion, and have different 5' upstream primer-specific portions. Thus, different primers (in the PCR amplification step (steps 4–5)) may be labeled with different fluorescent groups (Fam and Tet) to allow for distinction of products. This has the distinct advantage of allowing one to distinguish mononucleotide repeat polymorphisms based on fluorescent label instead of size, the latter being susceptible to false positives due to polymerase slippage. An array based detection scheme may also be used, where the upstream (allele-specific) probes have different 5' upstream primer-specific portions, and the different PCR primers contain different addressable array-specific portions which remain single stranded after a PCR reaction.

The LDR/exonuclease/PCR process described with reference to FIGS. **11** to **17** and the LDR/sequenase/uracil N-glycosylase/PCR process set forth in FIGS. **18**–**21** provide the ability to multiplex detect and then PCR amplify many different target sequences and to distinguish multiple single-base or sequence variations, all in a single reaction tube. This is achieved by combining the sensitivity of PCR with the selectivity of LDR. Since the selection of mutant sequences is mediated by LDR rather than PCR, the primary PCR/secondary PCR/LDR process is less susceptible to false-positive signal generation. In addition, the primary PCR/secondary PCR/LDR process allows detection of closely-clustered mutations, detection of single base or small insertions and deletions in small repeat sequences, quantitative detection of less than 1% mutations in high background of normal DNA, and detection of ligation product sequences using addressable arrays. Detection of single base or small insertions and deletions in small and medium repeat sequences may cause "stutter" when the primary amplification is PCR. No other currently-available technique can adequately solve this problem, especially when the target sequence containing the mononucleotide repeat polymorphism is present in a lower abundance than normal DNA. In fact, analysis of genomic mutations which involve repeat sequence changes is severely hampered by the PCR "stutter" problem. By using the LDR/PCR process of the present invention, it is possible to detect down to 1% mutations in a high background of normal DNA. The only relatively minor challenges presented by this process are that the mutations must be known and that 3 different enzymes/reaction conditions must be utilized.

III. Primary PCR/Secondary PCR Process

A third aspect of the present invention also involves a method for identifying two or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in one or more target nucleotide sequences. This method involves subjecting a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences to two successive polymerase chain reaction phases.

For the first polymerase chain reaction phase, one or more primary oligonucleotide primer groups are provided where each group comprises one or more primary oligonucleotide primer sets. Each set has a first oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primary-specific portion, and a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion. The first oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion and the second oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion. The oligonucleotide primers in a particular set are suitable for hybridization on complementary strands of a corresponding target nucleotide sequence to permit formation of a polymerase chain reaction product. However, there is a mismatch which interferes with formation of such a polymerase chain reaction product when the primary oligo-

nucleotide primers hybridize to any other nucleotide sequence present in the sample. The polymerase chain reaction products in a particular set may be distinguished from other polymerase chain reaction products with the same group or other groups. The primary oligonucleotide primers are blended with the sample and the polymerase to form a primary polymerase chain reaction mixture.

The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as described above. During the hybridization treatment, the target-specific portion of a primary oligonucleotide primer is hybridized to the target nucleotide sequences. In the extension treatment, the hybridized primary oligonucleotide primers are extended to form primary extension products complementary to the target nucleotide sequence to which the primary oligonucleotide primer is hybridized.

Although the upstream secondary primer-specific portions of a primary oligonucleotide primer set are not present on the target DNA, their sequences are copied by the second and subsequent cycles of the primary polymerase chain reaction phase. As a result, the primary extension products produced after the second cycle have the secondary primer-specific portions on their 5' ends and the complement of primer-specific portion on their 3' ends.

In the second polymerase chain reaction phase of this aspect of the present invention, one or a plurality of secondary oligonucleotide primer sets are provided. Each set has a first secondary primer having a detectable reporter label and containing the same sequence as the 5' upstream portion of a first primary oligonucleotide primer, -and a second secondary primer containing the same sequence as the 5' upstream primer of the second primary oligonucleotide primer from the same primary oligonucleotide primer set as the first primary oligonucleotide complementary to the first secondary primer. A set of secondary oligonucleotide primers amplify the primary extension products in a given group. The secondary oligonucleotide primers are blended with the primary extension products and the polymerase to form a secondary polymerase chain reaction mixture.

The secondary polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as described above. In the hybridization treatment, the secondary oligonucleotide primers are hybridized to the primary extension products, while the extension treatment causes the hybridized secondary oligonucleotide primers to be extended to form secondary extension products complementary to the primary extension products. After subjecting the secondary polymerase chain reaction mixture to the two or more polymerase chain reaction cycles, the labelled secondary extension products are detected. This indicates the presence of one or more target nucleotide sequences in the sample.

FIG. **22** is a flow diagram depicting a primary PCR/secondary PCR process, in accordance with the present invention, for detection of microsatellite repeats. In step 1 (i.e. the primary PCR phase), after DNA sample preparation, multiple exons are amplified using Taq polymerase under hot start conditions with oligonucleotide primers having a target-specific portion and a secondary primer-specific portion. Step 2 involves a secondary PCR phase where Taq polymerase is used to amplify the primary PCR extension products with oligonucleotide primers containing the same sequence as the secondary primer-specific portion of the primary PCR primers. The extension products resulting from the secondary PCR phase are subjected in step 3 to capillary electrophoresis or gel electrophoresis, followed by fluorescent quantification. The electrophoresis results in FIG. **22** indicate the presence of both alleles (i.e., chromosomes) containing RB1 and NM23 and loss of heterozygosity (i.e., loss of allele on one chromosome) for p53.

FIG. **23** is a schematic diagram depicting a primary PCR/secondary PCR process, according to the present invention, for detection of the loss of heterozygosity due to insertions and deletions in microsatellite repeats. The primary PCR phase in step 1 is initiated by denaturing the sample DNA at 94° C. Long PCR oligonucleotide primers, having 3' ends complementary to unique DNA surrounding microsatellite repeat sequences and 5' ends containing the same sequence as one of two primers utilized in the secondary PCR phase, are then caused to anneal to target DNA at 65° C. The primary PCR phase is carried out for 10–15 cycles. The long primers utilized in the primary PCR phase can be multiplexed as long as they do not amplify alleles with overlapping length ranges. These reactions must be carried out on tumor and corresponding normal DNA to identify informative (i.e heterozygous) loci. In step 2 (i.e secondary PCR amplification), primers complementary to the 5' ends of the primary PCR primers (one fluorescently labeled) are then used to amplify the primary PCR extension products at nearly equal efficiency. The secondary PCR extension products are then separated and analyzed by gel electrophoresis and an Applied Biosystems Inc. 373A DNA Sequencer using the Genescan 672 software package. Areas of loss of heterozygosity at informative loci are identified. The analysis in FIG. **23** shows the presence of both alleles (i.e., chromosomes) containing RB1 and NM23 and loss of heterozygosity (i.e., loss of allele on one chromosome) for p53.

IV. General Process Information

The ligase detection reaction is described generally in WO 90/17239 to Barany et al., F. Barany et al., *"Cloning, Overexpression and Nucleotide Sequence of a Thermostable DNA Ligase-encoding Gene," Gene,* 109:1–11 (1991), and F. Barany, "Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase," *Proc. Natl. Acad. Sci. USA,* 88:189–193 (1991), the disclosures of which are hereby incorporated by reference. In accordance with the present invention, the ligase detection reaction can use 2 sets of complementary oligonucleotides. This is known as the ligase chain reaction which is described in the 3 immediately preceding references, which are hereby incorporated by reference. Alternatively, the ligase detection reaction can involve a single cycle which is known as the oligonucleotide ligation assay. See Landegren, et al., "A Ligase-Mediated Gene Detection Technique," *Science* 241: 1077–80 (1988); Landegren, et al., "DNA Diagnostics—Molecular Techniques and Automation," *Science* 242:229–37 (1988); and U.S. Pat. No. 4,988,617 to Landegren, et al., which are hereby incorporated by reference

During ligase detection reaction phases, the denaturation treatment is carried out at a temperature of 80–105° C., while hybridization takes place at 50–85° C. Each cycle comprises a denaturation treatment and a thermal hybridization treatment which in total is from about one to five minutes long. Typically, the ligation detection reaction involves repeatedly denaturing and hybridizing for 2 to 50 cycles. The total time for the ligase detection reaction phase is 1 to 250 minutes.

The oligonucleotide probe sets or primers can be in the form of ribonucleotides, deoxynucleotides, modified ribo-

nucleotides, modified deoxyribonucleotides, modified phosphate-sugar-backbone oligonucleotides, nucleotide analogs, and mixtures thereof.

In one variation, the oligonucleotides of the oligonucleotide probe sets each have a hybridization or melting temperature (i.e. T_m) of 66–70° C. These oligonucleotides are 20–28 nucleotides long.

The oligonucleotide probe sets or primers, as noted above, have a reporter label suitable for detection. Useful labels include chromophores, fluorescent moieties, enzymes, antigens, heavy metals, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moieties, and electrochemical detecting moieties.

The polymerase chain reaction process is fully described in H. Erlich, et. al., "Recent Advances in the Polymerase Chain Reaction," *Science* 252: 1643–50 (1991); M. Innis, et. al., *PCR Protocols: A Guide to Methods and Applications*, Academic Press: New York (1990); and R. Saiki, et. al., "Primer-directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase," *Science* 239: 487–91 (1988), which are hereby incorporated by reference.

A particularly important aspect of the present invention is its capability to quantify the amount of target nucleotide sequence in a sample. This can be achieved in a number of ways by establishing standards which can be internal (i.e. where the standard establishing material is amplified and detected with the sample) or external (i.e. where the standard establishing material is not amplified, and is detected with the sample).

In accordance with one quantification method, the signal generated by the reporter label, resulting from capture of ligation product sequences produced from the sample being analyzed, are detected. The strength of this signal is compared to a calibration curve produced from signals generated by capture of ligation product sequences in samples with known amounts of target nucleotide sequence. As a result, the amount of target nucleotide sequence in the sample being analyzed can be determined. This techniques involves use of an external standard.

Another quantification method, in accordance with the present invention, relates to an internal standard. Here, a known amount of one or more marker target nucleotide sequences is added to the sample. In addition, a plurality of marker-specific oligonucleotide probe sets are added along with the ligase, the previously-discussed oligonucleotide probe sets, and the sample to a mixture. The marker-specific oligonucleotide probe sets have (1) a first oligonucleotide probe with a target-specific portion complementary to the marker target nucleotide sequence, and (2) a second oligonucleotide probe with a target-specific portion complementary to the marker target nucleotide sequence and a detectable reporter label. The oligonucleotide probes in a particular marker-specific oligonucleotide set are suitable for ligation together when hybridized adjacent to one another on a corresponding marker target nucleotide sequence. However, there is a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the sample or added marker sequences. The presence of ligation product sequences is identified by detection of reporter labels. The amount of target nucleotide sequences in the sample is then determined by comparing the amount of ligation product sequence generated from known amounts of marker target nucleotide sequences with the amount of other ligation product sequences.

Another quantification method, in accordance with the present invention involves, analysis of a sample containing two or more of a plurality of target nucleotide sequences with a plurality of sequence differences. Here, ligation product sequences corresponding to the target nucleotide sequences are detected and distinguished by any of the previously-discussed techniques. The relative amounts of the target nucleotide sequences in the sample are then quantified by comparing the relative amounts of captured ligation product sequences generated. This provides a quantitative measure of the relative level of the target nucleotide sequences in the sample.

The preferred thermostable ligase is that derived from *Thermus aquaticus*. This enzyme can be isolated from that organism. M. Takahashi, et al., "Thermophillic DNA Ligase," *J. Biol. Chem.* 259:10041–47 (1984), which is hereby incorporated by reference. Alternatively, it can be prepared recombinantly. Procedures for such isolation as well as the recombinant production of *Thermus aquaticus* ligase (as well as *Thermus themophilus* ligase) are disclosed in WO 90/17239 to Barany, et. al., and F. Barany, et al., "Cloning, Overexpression and Nucleotide Sequence of a Thermostable DNA-Ligase Encoding Gene," *Gene* 109: 1–11 (1991), which are hereby incorporated by reference. These references contain complete sequence information for this ligase as well as the encoding DNA. Other suitable ligases include *E. coli* ligase, T4 ligase, and *Pyococcus* ligase.

The ligation detection reaction mixture may include a carrier DNA, such as salmon sperm DNA.

The hybridization step in the ligase detection reaction, which is preferably a thermal hybridization treatment discriminates between nucleotide sequences based on a distinguishing nucleotide at the ligation junctions. The difference between the target nucleotide sequences can be, for example, a single nucleic acid base difference, a nucleic acid deletion, a nucleic acid insertion, or rearrangement. Such sequence differences involving more than one base can also be detected. Preferably, the oligonucleotide probe sets have substantially the same length so that they hybridize to target nucleotide sequences at substantially similar hybridization conditions. As a result, the process of the present invention is able to detect infectious diseases, genetic diseases, and cancer. It is also useful in environmental monitoring, forensics, and food science.

A wide variety of infectious diseases can be detected by the process of the present invention. Typically, these are caused by bacterial, viral, parasite, and fungal infectious agents. The resistance of various infectious agents to drugs can also be determined using the present invention.

Bacterial infectious agents which can be detected by the present invention include *Escherichia coli, Salmonella, Shigella, Klebsiella, Pseudomonas, Listeria monocytogenes, Mycobacterium tuberculosis, Mycobacterium avium-intracellulare, Yersinia, Francisella, Pasteurella, Brucella, Clostridia, Bordetella pertussis, Bacteroides, Staphylococcus aureus, Streptococcus pneumonia, B-Hemolytic strep., Corynebacteria, Legionella, Mycoplasma, Ureaplasma, Chlamydia, Neisseria gonorrhea, Neisseria meningitides, Hemophilus influenza, Enterococcus faecalis, Proteus vulgaris, Proteus mirabilis, Helicobacter pylori, Treponema palladium, Borrelia burgdorferi, Borrelia recurrentis, Rickettsial pathogens, Nocardia*, and *Acitnomycetes.*

Fungal infectious agents which can be detected by the present invention include *Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis, Paracoccidioides brasiliensis, Candida albicans, Aspergillus fumigautus, Phycomycetes* (*Rhizopus*), *Sporothrix schenckii, Chromomycosis*, and *Maduromycosis.*

Viral infectious agents which can be detected by the present invention include human immunodeficiency virus, human T-cell lymphocytotrophic virus, hepatitis viruses (e.g., Hepatitis B Virus and Hepatitis C Virus), Epstein-Barr Virus, cytomegalovirus, human papillomaviruses, orthomyxo viruses, paramyxo viruses, adenoviruses, corona viruses, rhabdo viruses, polio viruses, toga viruses, bunya viruses, arena viruses, rubella viruses, and reo viruses.

Parasitic agents which can be detected by the present invention include *Plasmodium falciparum, Plasmodium malaria, Plasmodium vivax, Plasmodium ovale, Onchoverva volvulus, Leishmania, Trypanosoma* spp., *Schistosoma* spp., *Entamoeba histolytica, Cryptosporidum, Giardia* spp., *Trichimonas* spp., *Balatidium coli, Wuchereria bancrofti, Toxoplasma* spp., *Enterobius vermicularis, Ascaris lumbricoides, Trichuris trichiura, Dracunculus medinesis, trematodes, Diphyllobothrium latum, Taenia* spp., *Pneumocystis carinii*, and *Necator americanis.*

The present invention is also useful for detection of drug resistance by infectious agents. For example, vancomycin-resistant *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae*, multi-drug resistant *Mycobacterium tuberculosis*, and AZT-resistant human immunodeficiency virus can all be identified with the present invention.

Genetic diseases can also be detected by the process of the present invention. This can be carried out by prenatal or post-natal screening for chromosomal and genetic aberrations or for genetic diseases. Examples of detectable genetic diseases include: 21 hydroxylase deficiency, cystic fibrosis, Fragile X Syndrome, Turner Syndrome, Duchenne Muscular Dystrophy, Down Syndrome or other trisomies, heart disease, single gene diseases, HLA typing, phenylketonuria, sickle cell anemia, Tay-Sachs Disease, thalassemia, Klinefelter Syndrome, Huntington Disease, autoimmune diseases, lipidosis, obesity defects, hemophilia, inborn errors of metabolism, and diabetes.

Cancers which can be detected by the process of the present invention generally involve oncogenes, tumor suppressor genes, or genes involved in DNA amplification, replication, recombination, or repair. Examples of these include: BRCA1 gene, p53 gene, APC gene, Her2/Neu amplification, Bcr/Ab1, K-ras gene, and human papillomavirus Types 16 and 18. Various aspects of the present invention can be used to identify amplifications, large deletions as well as point mutations and small deletions/insertions of the above genes in the following common human cancers: leukemia, colon cancer, breast cancer, lung cancer, prostate cancer, brain tumors, central nervous system tumors, bladder tumors, melanomas, liver cancer, osteosarcoma and other bone cancers, testicular and ovarian carcinomas, head and neck tumors, and cervical neoplasms.

In the area of environmental monitoring, the present invention can be used for detection, identification, and monitoring of pathogenic and indigenous microorganisms in natural and engineered ecosystems and microcosms such as in municipal waste water purification systems and water reservoirs or in polluted areas undergoing bioremediation. It is also possible to detect plasmids containing genes that can metabolize xenobiotics, to monitor specific target microorganisms in population dynamic studies, or either to detect, identify, or monitor genetically modified microorganisms in the environment and in industrial plants.

The present invention can also be used in a variety of forensic areas, including for human identification for military personnel and criminal investigation, paternity testing and family relation analysis, HLA compatibility typing, and screening blood, sperm, or transplantation organs for contamination.

In the food and feed industry, the present invention has a wide variety of applications. For example, it can be used for identification and characterization of production organisms such as yeast for production of beer, wine, cheese, yogurt, bread, etc. Another area of use is with regard to quality control and certification of products and processes (e.g., livestock, pasteurization, and meat processing) for contaminants. Other uses include the characterization of plants, bulbs, and seeds for breeding purposes, identification of the presence of plant-specific pathogens, and detection and identification of veterinary infections.

Desirably, the oligonucleotide probes are suitable for ligation together at a ligation junction when hybridized adjacent to one another on a corresponding target nucleotide sequence due to perfect complementarity at the ligation junction. However, when the oligonucleotide probes in the set are hybridized to any other nucleotide sequence present in the sample, there is a mismatch at a base at the ligation junction which interferes with ligation. Most preferably, the mismatch is at the base adjacent the 3' base at the ligation junction. Alternatively, the mismatch can be at the bases adjacent to bases at the ligation junction.

As noted supra, detection and quantification can be carried out using capillary or gel electrophoresis or on a solid support with an array capture oligonucleotides.

The use of capillary and gel electrophoresis for such purposes is well known. See e.g., Grossman, et. al., "High-density Multiplex Detection of Nucleic Acid Sequences: Oligonucleotide Ligation Assay and Sequence-coded Separation," *Nucl. Acids Res.* 22(21): 4527–34 (1994), which is hereby incorporated by reference.

The use of a solid support with an array of capture oligonucleotides is fully disclosed in pending provisional U.S. Patent Application Ser. No. 60/011,359, which is hereby incorporated by reference. When using such arrays, the oligonucleotide primers or probes used in the above-described coupled PCR and LDR phases, respectively, have an addressable array-specific portion. After the LDR or PCR phases are completed, the addressable array-specific portions for the products of such processes remain single stranded and are caused to hybridize to the capture oligonucleotides during a capture phase. C. Newton, et al., "The Production of PCR Products With 5' Single-Stranded Tails Using Primers That Incorporate Novel Phosphorarnidite Intermediates," *Nucl. Acids Res.* 21(5):1155–62 (1993), which is hereby incorporated by reference.

During the capture phase of the process, the mixture is contacted with the solid support at a temperature of 45–90° C. and for a time period of up to 60 minutes. Hybridizations may be accelerated by adding cations, volume exclusion or chaotropic agents. When an array consists of dozens to hundreds of addresses, it is important that the correct ligation product sequences have an opportunity to hybridize to the appropriate address. This may be achieved by the thermal motion of oligonucleotides at the high temperatures used, by mechanical movement of the fluid in contact with the array surface, or by moving the oligonucleotides across the array by electric fields. After hybridization, the array is washed sequentially with a low stringency wash buffer and then a high stringency wash buffer.

It is important to select capture oligonucleotides and addressable nucleotide sequences which will hybridize in a stable fashion. This requires that the oligonucleotide sets and the capture oligonucleotides be configured so that the oli-

gonucleotide sets hybridize to the target nucleotide sequences at a temperature less than that which the capture oligonucleotides hybridize to the addressable array-specific portions. Unless the oligonucleotides are designed in this fashion, false positive signals may result due to capture of adjacent unreacted oligonucleotides from the same oligonucleotide set which are hybridized to the target.

The capture oligonucleotides can be in the form of ribonucleotides, deoxyribonucleotides, modified ribonucleotides, modified deoxyribonucleotides, peptide nucleotide analogues, modified peptide nucleotide analogues, modified phosphate-sugar backbone oligonucleotides, nucleotide analogues, and mixtures thereof.

Where an array is utilized, the detection phase of the process involves scanning and identifying if LDR or PCR products have been produced and correlating the presence of such products to a presence or absence of the target nucleotide sequence in the test sample. Scanning can be carried out by scanning electron microscopy, confocal microscopy, charge-coupled device, scanning tunneling electron microscopy, infrared microscopy, atomic force microscopy, electrical conductance, and fluorescent or phosphor imaging. Correlating is carried out with a computer.

EXAMPLES

LDR/PCR Process

Example 1

Genomic DNA Preparation

Genomic DNA was prepared from the blood of two normal human volunteers, one male and one female, according to standard techniques. Briefly, approximately 12 ml of blood was obtained in EDTA-containing blood collection tubes. Red blood cells were lysed by mixing the blood samples with 4 volumes of lysis buffer (10 mM Tris pH 8.0, 10 mM EDTA). After 10 min on ice with occasional agitation, the suspensions were centrifuged and the supernatants were decanted. The white blood cell pellets were resuspended in 20 ml of lysis buffer, and the above process was repeated. Each cell pellet was then suspended in 15 ml of digestion buffer (50 mM Tris pH 8.0, 5 mM EDTA, 100 mM NaCl, 1% SDS) and 3 mg (0.2 mg/ml) of proteinase K was added. The cells were digested at 37° C. for 5 hours. The digests were extracted twice with equal volumes of phenol, then once with equal volumes of a 1:1 phenol:chloroform mixture and finally once with equal volumes of chloroform, each time centrifuging the mixture and removing the aqueous phase for the next extraction. After the final extraction and removing the aqueous phases, one tenth volume of 3 M sodium acetate, pH 6.5, was added. Two volumes of ice cold 100% EtOH were then added to each solution to precipitate the genomic DNAs, which were spooled out of solution on glass pipettes. The DNA precipitates were washed twice in 0.75 ml volumes of 70% EtOH, briefly centrifuging each time to allow removal of the supernatants. After removing the supernatants for the second time, the remaining EtOH was allowed to evaporate and the DNA was suspended in 0.5 ml of TE (10 mM Tri-HCl pH 8.0 containing 1 mM EDTA) solution. A fifth dilution of each DNA solution was also prepared in TE.

To determine the concentrations of the one fifth DNA solutions, 1, 2, and 4 μl aliquots of each were loaded on a 1% agarose gel with a known amount of HindIII digested lambda DNA as a control. The gel was run at 150 Volts for 2 hours with ethidium bromide in the electrophoresis buffer. After photographing the gel and comparing the intensities of the DNA bands, the one fifth dilutions were judged to have concentrations of approximately 100 ng/ml. DNA solutions extracted from various tumor cell lines were the generous gifts of other laboratories. The concentrations of these solutions were checked in a similar fashion and solutions of 100 ng/ml in TE were prepared.

To digest the genomic DNAs with Taq I, 25 μl of the 100 ng/μl solutions was mixed with 5 μl of 10X medium salt buffer (0.5 M NaCl, 0.1 M $MgCl_2$, 0.1 M Tris, pH 8.0), 20 μl of water-ME (i.e. water containing 6mM ME (i.e., mercaptoethanol)), and 400 U of Taq I restriction endonuclease. The digests were covered with mineral oil and incubated at 65° C. for 1 hour. The reactions were stopped by adding 1.2 μl of 500 mM EDTA and heating the specimens to 85° C. for 10 min. Complete digestion of the DNAs was checked by electrophoresing aliquots on a 1% agarose gel.

Example 2

Oligonucleotide Preparation for LDR Probes and PCR Primers

All oligonucleotides were synthesized on a 394A DNA Synthesizer (Applied Biosystems Division of Perkin-Elmer Corp., Foster City, Calif.). Oligonucleotides labeled with 6-FAM were synthesized using the manufacturer's suggested modifications to the synthesis cycle (Applied Biosystems Inc., 1994) and were subsequently deprotected at 55° C. for 4 hr. LDR oligonucleotides were purified by ethanol precipitation after overnight deprotection at 55° C. The primer-specific portions of the oligonucleotides used for PCR amplification were purified by polyacrylamide gel electrophoresis on 10% acrylamide/7M urea gels. Oligonucleotides were visualized after electrophoresis by UV shadowing against a lightening screen and excised from the gel (Applied Biosystems Inc., 1992). They were then eluted overnight at 64° C. in TNE (i.e. Tris-sodium EDTA) buffer (100 mM Tris/HCl pH 8.0 containing 500 mM NaCl and 5 mM EDTA) and recovered from the eluate using Sep Pak cartridges (Millipore Corp, Milford, Mass.) following the manufacture's instructions.

Oligonucleotides were resuspended in 100 μl 1 TE (i.e. 10 mM Tri-HCl pH 8.0 containing 1 mM EDTA). Typical concentrations of these original LDR probe solutions are about 1 μg/μl or approximately 74 pm/μl as determined by the following formula:

$$[\text{concentration } (\mu g/\mu l)\times 10^6]/[\text{length (nt)}\times 325]=\text{pm}/\mu l$$

The concentrations of the LDR probes are given in Table 1. The concentrations of oligonucleotides complementary to the oligonucleotide probes of the ligase detection reaction were higher. ZipALg1F was 3.75 μg/μl and ZipBLg2R was 2.01 μg/μl or 524 pm/μl and 281 pm/μl, respectively, as determined by the formula above.

TABLE 1

Primer	Length	μg/μl	pm/μl	Vol = 100 pm	l = 200 pm
G6PDEx6-3L	48 nt	0.86	55.1	1.81 μl	3.63 μl
G6PDEx6-4R	48	0.65	41.7	2.4	4.8
ErbBEx1-5L	48	0.95	60.9	1.64	3.28
ErbBEx1-5R	48	1.4025	89.9	1.11	2.22
Int2Ex3-7L	50	1.6005	98.5	1.02	2.03

TABLE 1-continued

Primer	Length	µg/µl	pm/µl	Vol = 100 pm	l = 200 pm
Int2Ex3-8R	46	1.306	87.4	1.14	2.29
p53Ex8-9L	2	1.036	61.3	1.63	3.26
p53Ex8-10R	44	1.164	81.4	1.23	2.46
SODEx3-11L	49	1.287	80.8	1.24	2.48
SODEx3-12R	47	1.2045	78.9	1.27	2.53

As a prerequisite for the LDR phase, the downstream LDR oligonucleotides probes were phosphorylated with T4 polynucleotide kinase. Aliquots of the 5 downstream oligonucleotides equivalent to 200 pm (see Table 1) were combined with 10 µl of 10×kinase buffer (500 mM Tris/HCl pH 8.0, 100 mnM $MgCl_2$), 10 µl of 10 mM ATP, 20 U T4 kinase, and sufficient water-ME to give a final volume of 100 µl. Phosphorylation was carried out at 37° C. for 30 min followed by incubation for 10 min at 85° C. to inactivate the T4 enzyme. The resulting concentration of the kinased LDR probe solution was 2 pm/µl or 2000 fm/µl in each probe.

The kinase reaction is summarized as follows:

4.8	µl	G6PDEx6-4R
2.2	µl	ErbBEx1-5R
2.3	µl	Int2Ex3-8R
2.5	µl	p53Ex8-10R
2.5	µl	SODEx3-12R
10	µl	10× Kinase Buffer
10	µl	10 mMATP
65.7	µl	HOH + ME
100	µl	Total
+ 2	µl	= 20 units T4 Kinase

37° C. for 30 min.
Heat kill kinase, 85° C., 10 min.
Final concentration = 2 pm/µl = 2000 fm/µl

The solutions of the LDR and PCR oligonucleotides were adjusted to convenient concentrations. The kinased LDR probe solution was diluted fourfold in water to yield a concentration of 500 fm/µl. A solution of the upstream LDR probes was made by combining volumes of the probes equivalent to 200 pm (see Table 1) with sufficient water to give a final volume of 400 µl. This created a solution 500 fm/µl in each of the upstream LDR probes. Aliquots (20 µl) of the kinased and unkinased LDR probes were frozen for subsequent use. Standard solutions of the PCR primers (10 pm/µl) were prepared from their original solutions by combining 9.5 µl of ZipALg1F and 17.8 µl of ZipBLg2R with sufficient water to achieve a total volume of 500 µl. These solutions were frozen for use in the LDR/PCR process.

Unkinased probes were prepared according to the following:

200 pm	ea Primer
3.62 µl	G6PDEx6-3L
3.28 µl	ErbBEx1-5L
2.04 µl	Int2Ex3-7L
3.26 µl	p53Ex8-9L
2.48 µl	SODEx3-11L
385.32 µl	HOH
400 µl	Total Vol

Final concentration=0.5 pm/µl 1=500 fm/µl

TABLE 2

		Sequences				
		Probe (length in nt)				
Gene	Location	Upstream		Downstream		Ligation Position
erb	17q12–q21	erbBEx1-5L	(48)	erbBEx1-6R	(48)	exon "1" P40
G6PD	Xq28	G6PDEx6-3L	(48)	G6PDEx6-4R	(48)	exon 6 W1145
Int2	11q13	Int2Ex3-7L	(50)	Int2Ex3-8R	(46)	exon 3 W135
p53	17p13.1	p53Ex8-9L	(52)	p53Ex8-10R	(44)	exon 8 P51
SOD	21q22.1	SODEx3-11L	(49)	SODEx3-12R	(47)	exon 3 P355

FIG. **24** shows the design of LDR oligonucleotide probes for quantification of gene amplifications and deletions in the LDR/PCR process. These oligonucleotide probes were designed to recognize exon 8 in the p53 tumor suppressor gene (on chromosome 17p), exon 3 of int-2 (on chromosome 11q), an internal exon in HER-2/neu (i.e. HER-2/neu/erbB oncogene) (on chromosome 17q), exon 3 in SOD (i.e. super oxide dimutase) (on chromosome 21q), and exon 6 in G6PD (i.e. glucose 6-phosphate dehydrogenase) (on chromosome Xq). Each pair of LDR oligonucleotide probes has the following features: (i) The left oligonucleotide probe contains from 5' to 3' an 18 base sequence identical to the fluorescently labeled secondary oligonucleotide primer (black bar), an "adjustment sequence" (white bar), and a target-specific sequence of from 22 to 28 bases with a T_m of 75° C. (patterned bar); (ii) The right oligonucleotide probe contains from 5' to 3' a target-specific sequence of 20–25 bases with a T_m of 75° C. (patterned bar), a single HaeIII or HinP1I restriction site at slightly different positions within the target-specific sequence, and an "adjustment sequence" (white bars). The two oligonucleotide probes are designed such that their combined length is exactly 96 bases, with 50 G+C bases and 46 A+T bases. The position of each unique restriction site generates a product which differs by at least 2 bases from the other products. Each oligonucleotide probe set has an exon-specific region chosen to ligate the junction sequence of (A, T)C C(A, T). This junction sequence corresponds to either a proline residue (codon CCN) or the complementary sequence of a tryptophan residue (TGG). These sequences were chosen to minimize differences in ligation rates and the chance of a polymorphism at the ligation junction.

LDR Probe Sequences

G6PDEx6-3L (SEQ. ID. NO. 1)

5'CAC GCT ATC CCG TTA GAC ATT GTC AAG CAG GCG ATG TTG TCC CGG TTC 3'

G6PDEx6-4R (SEQ. ID. NO. 2)

5'CAG ATG GGG CCG AAG ATC CTG TTA TTG ATA CAT AGT GCG GTA GTT GGC 3'

erbBEx1-5L (SEQ. ID. NO. 3)

5'CAC GCT ATC CCG TTA GAC ATC GCC CTG ATG GGG AGA ATG TGA AAA TTC 3'

erbBEx1-6R (SEQ. ID. NO. 4)

5'CAG TGG CCA TCA AAG TGT TGA GGG AGC GTA CAT AGT GCG GTA GTT GGC 3'

Int2Ex3-7L (SEQ. ID. NO. 5)

5'CAC GCT ATC CCG TTA GAC ATT CAT AAC CCT TGC CGT TCA CAG ACA CGT AC 3'

Int2Ex3-8R (SEQ. ID. NO. 6)

5'CAC AGT CTC TCG GCG CTG GGC AAT AAT ACA TAG TGC GGT AGT TGG C 3'

p53Ex8-9L (SEQ. ID. NO. 7)

5'CAC GCT ATC CCG TTA GAC ATC TTA GTA ATT GAG GTG CGT GTT TGT GCC TGT C 3'

p53Ex8-10R (SEQ. ID. NO. 8)

5'CTG GGA GAG ACC GGC GCA CAT TAC TAC ATA GTG CGG TAG TTG GC 3'

SODEx-3-11L (SEQ. ID. NO. 9)

5'CAC GCT ATC CCG TTA GAC ATC TGT ACC AGT GCA GGT CCT CAC TTT AAT C 3'

SODEx-3-12R (SEQ. ID. NO. 10)

5'CTC TAT CCA GAA AAC ACG GTG GGC CGC TAC ATA GTG CGG TAG TTG GC 3'

PCR Primers:

ZipALg1F 5' Fam-GGA GCA CGC TAT CCC GTT AGA C 3' (SEQ. ID. NO. 11) (Tm = 71° C.)

ZipBLg2R 5' CGC TGC CAA CTA CCG CAC TAT G 3' (SEQ. ID. NO. 12) (Tm = 72° C.)

(Underlined sequences are common between LDR probes and ZipALg1F or the complement of ZipBLg2R.)

Example 3

Buffers and Reagents

A. LDR Buffers/Reagents—the following LDR buffers and reagents were selected:

10×ST ligase buffer (0.2 M Tris pH 8.5, 0.1 M $MgCl_2$) [This was also tested with Tris at pH 7.6.]

10×TT ligase buffer (0.2 M Tris pH 7.6, 0.5 M KCl, 0.1 M $MgCl_2$, 5mM EDTA)

NAD (10 mM)

DTT (200 mM)

LDR primer solution containing one tenth concentration of each of the LDR primer mixtures (50 fm of each LDR primer per μl)

Tth DNA Ligase (625 U/μl)

PCR Buffers/Reagents—the following PCR buffers and reagents were selected:

10×Stoffel buffer (0.1 M KCl, 0.1M Tris-HCl pH 8.3 Perkin Elmer)

dNTP solution (100 MM total, 25 mM of each dNTP Perkin Elmer), diluted 5 fold in

dHOH to a final concentration of 5 mM of each dNTP

ZipALg1F (10 pm/μl)

ZipBLg2R (10 pm/μl)

Example 4

LDR/PCR Process

Four LDR/PCR processes were performed for each DNA to be tested with reaction tubes PCR amplified to 22, 24, 26, and 30 cycles to assure that one reaction would be halted in the exponential phase. Each LDR reaction (20 μl) was thermal cycled and then a PCR mix (30 μl) containing primers with a portion complementary to the primer-specific portion of the LDR probes was added to each specimen to allow exponential amplification. To minimize differences between reaction tubes, master mixes of LDR and PCR reagents were made.

A master mix of LDR reagents was constructed with a volume sufficient for all reaction tubes. Proportions and volumes for a single reaction were as follows:

Reagent	Volume
10X ST Ligase Buffer	2 µl
NAD (10 mM)	2 µl
DTT (200 mM)	1 µl
dHOH_	5 µl
Total	10 µl
Tth DNA Ligase	0.2 µl (=125 U)

Mixes of target DNA and LDR probes for each reaction tube were constructed with the following proportions:

Reagent	Volume
DNA (TaqI digested)	1 µl (=50 ng)
LDR Probe Mix	4 µl (200 fm each primer)
dHOH_	5 µl
Total	10 µl

For each reaction, 10 µl was placed in a thin-walled PCR tube, rapidly mixed with 10 µl of LDR reagent mix (including ligase), overlayed with mineral oil, and placed in a Perkin Elmer 9600 thermal cycler.

LDR was initiated by holding at 96° C. for 2 minutes to denature the DNA followed by 10 cycles of 94° C. for 30 seconds and 65° C. for 4 minutes.

PCR reagent mixes for each reaction tube were constructed with the following proportions:

Reagent	Volume	
10X Stoffel buffer	5 µl	
dNTP solution	8 µl	(=0.8 mM each dNTP in final reaction)
ZipALg1F (10 pm/µl)	2.5 µl	(=25 pm per reaction)
ZipBLg2R (10 pm/µl)	2.5 µl	(=25 pm per reaction)
dHOH	12 µl	
Total	30 µl	
Stoffel Fragment	0.25 µl	(=2.5 U)

At the completion of the LDR reaction, the tubes were held at 94° C., while 30 ml of PCR reagent mix (including Stoffel fragment) were added to each tube. PCR amplification was accomplished by thermal cycling at 94° C. for 15 seconds followed by 60° C. for 50 seconds. At 22, 24, 26, and 30 cycles, respectively, one of four identical reaction tubes of each DNA specimen was removed and quenched in a slurry of dry ice and ETOH.

Example 5

Agarose Gel Evaluation

Ten microliter aliquots of the 26 and 30 cycle reaction specimens were evaluated on a 2% agarose gel. Ethidium bromide staining revealed bands of the expected size (104 bp).

Example 6

Digestion of Products, Preparation of Dilutions, and Loading on GeneScanner

To separate the gene-specific LDR/PCR products, 10 µl aliquots of the 22, 24, and 26 cycle reactions were digested by adding 10 µl of a solution containing 5 U each of HaeIII and HinP1I restriction enzymes (both from New England BioLabs), 2 µl of 10× restriction enzyme buffer number 2 (New England BioLabs), and 8 µl of dHOH (i.e. distilled water). The digests were incubated at 37° C. for one hour and then stopped by the addition of 1 µl of 0.5 M EDTA, pH 8.0. The restriction digests were a one half dilution of the original LDR/PCR products. A 10 fold dilution of each sample was also prepared by adding 5 µl of each restriction digest to 20 µl of TE buffer.

Before loading samples on the ABI 373A DNA Sequencer (Applied Biosystems) a 1:5 mixture of 50 mM EDTA pH 8.0 containing 2% blue dextran and de-ionized formamide was made. To 5 µl of the EDTA-Blue Dextran solution, 5 µl of digested LDR/PCR product dilution and 1 µl of GENESCAN 1000 ROX marker (Applied Biosystems) were added. These solutions were heated to 85° C. for 10 minutes and snap chilled on ice, before 5.5 µl were loaded on the denaturing gel.

Samples were analyzed in an Applied Biosystems 373A DNA sequencer on a 0.4 mm thick, 10% polyacrylamide/7M urea gel with a well-to-read distance of 12 cm. The gel matrix was buffered with 1.2×TBE (106 mM Tris-borate and 2.4 mM EDTA pH 8.3) and the electrophoresis chamber buffer contained 0.6×TBE (53.4 mM Tris-borate and 1.2 mM EDTA pH 8.3). The gel was pre-run prior to sample loading at 1600 V for 30 minutes with the electrode polarity reversed (anode in the chamber with sample wells at the top of the gel). After loading, the gene-specific LDR/PCR products were electrophoresed at 1200 V and the primary data was captured using the ABI 672 Data Collection Software V1.1 (Applied Biosystems)

Using the ABI 672 GeneScan Analysis Software Vl.2.2 (Applied Biosystems), the resulting data were displayed as electropherograms, with peak heights and peak areas calculated.

In the normal female, the ErbB2 peak is lower, and the p53 peak is slightly lower than the remaining 3 peaks. See FIGS. **25**A–D. In different experiments, it was observed that the ErbB2 peak is always lower, the G6PD, Int-2, p53, and SOD peak areas would vary somewhat, but all 5 peaks would retain the same relative profile from one sample to the next for a given experiment. When comparing male with female DNA, the G6PD peak was about half the area of other peaks, consistent with a single X-chromosome in males, while the other peaks were essentially the same. The ErB2 peak for the NM10 Breast Cancer cell line is slightly elevated, while that in cell line SKBR3 is several fold greater than the normal female control, reflecting the known ErbB-2 gene amplification in these two cell lines. In addition, cell line NM10 appears to have undergone LOH (i.e. a loss of heterozygosity) of p53, while cell line SKBR3 appears to have undergone LOH of G6PD and p53. Some of the cells in cell line SKBR3 may have lost both copies of the p53 gene. Repeating these amplifications in the absence of the ErbB-2 primers was used to confirm the presence of these additional gene deletions (see below).

These results can be quantified by comparing the ratio of peak areas in each peak to a standard (the SOD peak area) for that experiment. The raw data and ratio of peak areas are given below:

TABLE 3

Raw Peak Area Data

	Genes				
	ErB	G6PD	Int2	p53	SOD
Male	9954	21525	45688	36346	62506
Female	8340	39309	39344	30270	54665
NM10	20096	55483	67083	17364	84339
SKBR3	106650	19120	50103	2888	48119

TABLE 4

Ratio of Peak Areas to SOD Peak Area

	ErbB/SOD	G6PD/SOD	Int2/SOD	p53/SOD
Male	0.16	0.34	0.73	0.58
Female	0.15	0.72	0.72	0.55
NM10	0.24	0.66	0.80	0.21
SKBR3	2.22	0.40	1.04	0.06

Although the ratios differ for each gene, (due to different efficiencies of LDR/PCR for each gene,) the ratios are generally consistent between the male and female sample, except for the G6PD/SOD ratio. The G6PD for the female is about twice the value as the male, accurately reflecting the presence of two and one X chromosome, respectively. One can quantify the amount of ErbB2 amplification by comparing the ratio of peak area ratios between normal DNA and cancer cell lines.

TABLE 5

Ratio of Peak Areas Ratios

	ErbB/2	G6PD	Int2	p53
Female/Male	0.96	2.09	0.98	0.95
NM10/Male	1.50	1.91	1.09	0.35
SKBR3/Male	13.92	1.15	1.42	0.10

From these ratios, it can be determined that the normal male and female have the same number of genes on chromosomes 17q (ErbB), 17p (p53), and 11q (Int 2), but that the female has twice as many G6PD genes, or X chromosomes. Likewise, cell line NM10 showed slight amplification of the ErbB-2 gene, and LOH at p53, while cell line SKBR3 shows significant amplification of the ErbB-2 gene, LOH at G6PD and p53. To confirm additional gene amplifications and deletions, primer pairs causing massive amplifications may be removed from the LDR/PCR reaction (see below).

In the normal female, the ErbB2 peak is lower than the remaining 4 peaks. In different experiments, it was observed that the G6PD, Int-2, p53, and SOD peak areas would vary somewhat, but would retain the same relative profile from one sample to the next. See FIGS. **26**A–C. The ErbB2 peak was consistently lower, and slight shoulders were observed on the G6PD and SOD peaks, for unknown reasons. The ErbB-2 peak in both cell line samples is several fold greater than the normal female control, reflecting the known ErbB-2 gene amplification in these two cell lines. In addition, the ZR-75-30 line appears to show LOH of p53, while the SKGT-2 cell line appears to have a slight amplification of the Int-2 region. By repeating these LDR/PCR experiments in the absence of the ErbB-2 primers, it was demonstrated that these results are not artifacts of the massive levels of ErbB-2 amplification. See FIGS. **27**A–C. Both gene amplifications and deletions for multiple genes using the LDR/PCR format have been demonstrated. See FIGS. **26**A–C and **27**A–C.

Again, these results can be quantified by comparing the ratio of peak areas in each peak to a standard (the SOD peak area) for that experiment. The raw data and ratio of peak areas are given below:

TABLE 6

Raw Peak Area Data

	Genes				
	ErbB	G6PD	Int2	p53	SOD
Female; 4 Primer Sets	NA	9577	8581	9139	8128
ZR7530; 4 Primer Sets	NA	8452	7904	4168	7996
SKGT2; 4 Primer Sets	NA	15915	28614	13116	12478
Female; 5 Primer Sets	3955	9436	8066	9304	8848
ZR7530; 5 Primer Sets	66748	11105	8812	4163	9303
SKGT2; 5 Primer Sets	263254	21877	31887	13630	13480

TABLE 7

Ratio of Peak Areas to SOD Peak Area

	ErbB/SOD	G6PD/SOD	Int2/SOD	p53/SOD
Female; 4 Primer Sets	NA	1.18	1.06	1.12
ZR7530; 4 Primer Sets	NA	1.06	0.99	0.52
SKGT2; 4 Primer Sets	NA	1.28	2.29	1.05
Female; 5 Primer Sets	0.45	1.97	0.91	1.05
ZR7530; 5 Primer Sets	7.17	1.19	0.95	0.45
SKGT2; 5 Primer Sets	19.53	1.62	2.37	1.01

The ratios are remarkably consistent between the four primer set and the five primer set experiments. The only exception is the G6PD peak for the SKGT2 cell line, where the huge peak for ErbB-2 may have added to the G6PD peak.

One can quantify the amount of ErbB2 and Int-2 amplification as well as p53 deletion by comparing the ratio of peak area ratios between normal DNA and cancer cell lines, as shown in Table 8. In addition, the ratios from using 4 sets of primers can be compared with 5 sets of primers to ascertain the internal consistency of this technique.

TABLE 8

Ratio of Peak Area Ratios

	ErbB	G6PD	Int2	p53
Female; 4/5	NA	1.10	1.16	1.07
ZR7530; 4/5	NA	0.89	1.04	1.16
SKGT2; 4/5	NA	0.79	0.97	1.04
ZR7530/Female; 4/4	NA	0.90	0.94	0.46
ZR7530; Female; 5/5	16.05	1.12	1.04	0.43
SKGT2/Female; 4/4	NA	1.08	2.17	0.93
SKGT2; Female; 5/5	43.69	1.52	2.59	0.96

The values for the top half of Table 8 should all be close to 1.0 if the LDR/PCR technique is internally consistent, when using 4 or 5 primers. All values are very close to 1.0. Again, the value for G6PD for SKGT2 is a bit low for the reasons mentioned.

The values on the bottom half of Table 8 show the extent of ErbB-2 amplification. The numbers are quite consistent for the 4 primer and 5 primer amplifications (with the exception of SKGT2-G6PD noted above). The ZR7530 cell line demonstrates a clear LOH for p53, while the SKGT2 cell line shows amplification of the Int-2 region, and both p53 genes present.

Primary PCRISecondary PCR/LDR Process

Example 7

Oligonucleotide Synthesis

Oligonucleotides were assembled by standard phosphoramidite chemistry on an Expedite DNA synthesizer (Perseptive Biosystems, Framingham, Mass.). Oligonucleotides 5'-end labeled with 6-FAM, TET, and HEX were synthesized using the appropriate dye phosphoramidites (Perkin Elmer-Applied Biosystems) and purified with Oligonucleotide Purification Cartridges (Perkin Elmer-Applied Biosystems) following the manufacturer's protocol (Applied Biosystems Division-Perkin Elmer Corp., "Synthesis and Purification of Fluorescently Labeled Oligonucleotides Using Dye Phosphoramidites," *User Bulletin*, number 78, Applied Biosystems Division, Foster City, Calif., (1994)), which is hereby incorporated by reference. All oligonucleotides were checked for purity on an Applied Biosystems 270-HT capillary electrophoresis instrument using a mPAGE-3 column (J&W Scientific, Folsom, Calif.). Only oligonucleotides that were greater than 95% pure were used for the experiments. Oligonucleotides were resuspended in 250 ml TE (10 mM Tris/HCl and 5 mM EDTA pH 8.0). Typical concentrations were 300–500 mM for crude stock solutions and 100–200 mM for OPC (i.e. Oligonucleotide Purification Columns available from Applied Biosystems) purified stock solutions. For PCR and LDR, oligonucleotides were diluted to working solutions of 10 mM (10 pmoles/ml) or 5 mM (5 pmoles/ml).

Example 8

Phosphorylation of LDR Oligonucleotides

The 12 LDR common oligonucleotides were phosphorylated at the 5' end to permit ligation to the fluorescent labeled oligonucleotides. The oligonucleotides are shown below in Table 9.

TABLE 9

LDR Oligonucleotide Sequences

Locus	Allele-Specific Oligonucleotide (5'-->3')	Common Oligonucleotide (5'-->3')
1	FAM-AGCTTCAATGATGAGAACCTGC (SEQ. ID. NO. 13)	P-GCATAGTGGTGGCTGACCTGTTCATAT (SEQ. ID. NO. 14)
	TET-AGCTTCAATGATGAGAACCTGT (SEQ. ID. NO. 15)	
2	FAM-CTCCATGGGCCCAGCC (SEQ. ID. NO. 16)	P-AGCACTGGTGCCCTGTGAG (SEQ. ID. NO. 17)
	TET-CTCCATGGGCCCAGCT (SEQ. ID. NO. 18)	
3	FAM-GGGGACAGCCATGCACTGA (SEQ. ID. NO. 19)	P-GCCTCTGGTAGCCTTTCAACCATA (SEQ. ID. NO. 20)
	TET-GGGGACAGCCATGCACTGC (SEQ. ID. NO. 21)	
4	FAMTTAGAAATCATCAAGCCTAGGTCAT (SEQ. ID. NO. 22)	P-CACCTTTTAGCTTCCTGAGCAATGAT (SEQ. ID. NO. 23)
	TET-TTAGAAATCATCAAGCCTAGGTCAG (SEQ. ID. NO. 24)	
5	HEX-GGTTGTATTTGTCACCATATTAATTA (SEQ. ID. NO. 25)	P-ATTTTTCTCTATTGTTTTCATCTTTCAGGA (SEQ. ID. NO. 26)
	HEX-ATGGTTGTATTTGTCACCATATTAATTG (SEQ. ID. NO. 27)	
6	FAM-GGGCCAAGAAGGTATCTACCA (SEQ. ID. NO. 28)	P-ATAGTGTCTATTAGGCATTTGAAAATGTGTAT (SEQ. ID. NO. 29)
	TET-GGGCCAAGAAGGTATCTACCG (SEQ. ID. NO. 30)	
7	FAM-ACACAGCAGCTTACTCCAGAGG (SEQ. ID. NO. 31)	P-TCAAGTCCAAGGCCATTGGCTTATA (SEQ. ID. NO. 32)
	TET-ACACAGCAGCTTACTCCAGAGA (SEQ. ID. NO. 33)	
8	FAM-CCAGCAAAGAGAAAAGAAGGG (SEQ. ID. NO. 34)	P-CCCCAGAAATCACAGGTGGGCTAT (SEQ. ID. NO. 35)

TABLE 9-continued

LDR Oligonucleotide Sequences

Locus	Allele-Specific Oligonucleotide (5'-->3')	Common Oligonucleotide (5'-->3')
	TET-CCAGCAAAGAGAAAAGAAGGA (SEQ. ID. NO. 36)	
9	FAM-ATGATATTAGAGCTCACTCATGTCCA (SEQ. ID. NO. 37)	P-TCAGTTTGGAAAAAGACAAAGAATTCTTT (SEQ. ID. NO. 38)
	TET-ATGATATTAGAGCTCACTCATGTCCG (SEQ. ID. NO. 39)	
10	HEX-TGCTGTCTTCCAGGAATCTGTT (SEQ. ID. NO. 40)	P-CAACTCTCTCGAAGCCATGTTCACAA (SEQ. ID. NO. 41)
	HEX-ATTGCTGTCTTCCAGGAATCTGTG (SEQ. ID. NO. 42)	
11	HEX-GGACATAGTGACCGTGCAGGTC (SEQ. ID. NO. 43)	P-CTTCCCCAGTGTGAGTGCCGTA (SEQ. ID. NO. 44)
	HEX-ATGGACATAGTGACCGTGCAGGTT (SEQ. ID. NO. 45)	
12	HEX-CTATGACACCGTCATCAGCAGG (SEQ. ID. NO. 46)	P-GACATCCAGGCCCCCGAC (SEQ. ID. NO. 47)
	HEX-TACTATGACACCGTCATCAGCAGA (SEQ. ID. NO. 48)	

The allele-specific oligonucleotides are 5' end labeled with either FAM, TET, or HEX. All the common oligonucleotides are phosphorylated at the 5' end. Underline denotes tails that are not complementary to the target sequence. LDR primer sets were designed in two ways: (i) allele-specific primers were of the same length but contained either FAM or TET label; or (ii) the allele-specific primers were both labeled with HEX but differed in length by two bases.

This was accomplished either during the synthesis with Phosphate-ON (Clontech Laboratories, Palo Alto, Calif.) according to the manufacturer's instructions or post-synthesis, using T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, Ind.). In the latter, a common oligomer was diluted into 50 μl of kinase buffer (50 mM Tris/HCl, 10 mM $MgCl_2$, and 1 mM ATP) to a final concentration of 1 mM (500 pmol in 50 μl). Ten units of T4 kinase was added, and the reaction was incubated at 37° C. for 30 min. The T4 kinase was inactivated by heating at 95° C. for 10 min.

The kinase reaction was carried out as follows:

10 μl	50 mM common oligo
5 μl	10× kinase buffer
5 μl	10 mM ATP
30 μl	H20
50 μl	Total
+ 1 μl	T4 Kinase (10 units)

37° C. for 30 min.
95° C. for 10 min.
Final concentration = 10 mM

Example 9

Multiplex PCR Amplification

Twelve gene regions (2–13) were chosen for simultaneous PCR amplification based on information available in the Human Genome Database, as shown below in Table 10.

TABLE 10

List of Polymorphic Sites Analyzed

Site Number	Locus Symbol	Locus Name	Chr. Location	Nucleotide Position	Variation	Het.	Ref.
1	CYP2D6	cytochrome P450IID6	22q13.1	4469 (M33388)	C, T	.38	2
2	AT3	antithrombin III	1q23–q25.1	7987 (X68793)	C, T	.46	3
3	C6	complement component C6	5p14–p12	185 (X72179)	A, C	.47	4

TABLE 10-continued

List of Polymorphic Sites Analyzed

Site Number	Locus Symbol	Locus Name	Chr. Location	Nucleotide Position	Variation	Het.	Ref.
4	IL1A	interleukin 1 alpha	2q13	6282 (X03833)	T, G	.34	5
5	NF1	neurofibromatosis	17q11.2	63683 (L05367)	A, G	.47	6
6	ALDOB	aldolase B	9q22.3–q31	1087 (M15656)	A, G	.50	7
7	A2M	alpha 2 macroglobulin	12p13.3–p12.3	153 (X68731)	G, A	.42	8
8	IGF2	insulin growth factor	11p15.5	820 (X07868)	G, A	.46	9
9	PROS1	protein S alpha	3p11-cen	183 (M36564)	A, G	.50	10
10	LIPC	triglyceride lipase	15q21–q23	113 (M29189)	T, G	.49	11
11	CD18	integrin B-2 subunit	21q22.3	109 (X64081)	C, T	.50	12
12	LDLR	low density lipoprotein receptor	19p13.2	70 (L00344)	G, A	.50	13

The site numbers are specific single point variations located within the respective genes. All variations indicated are defined on the sense strand. Genbank accession numbers are indicated in parentheses. Chr., chromosome; Het., heterozygosity.

References in Table 10

2 M. Armstrong, et al., "A Polymorphic Cfo I Site In Exon 6 of the Human Cytochrome P450 CYPD6 Gene Detected by the Polymerase Chain Reaction," Human Genetics 91: 616–17 (1993), which is hereby incorporated by reference.

3 S. C. Bock, et al., "Antithrombin III Utah: Proline-407 to Leucine Mutation in a Highly Conserved Region Near the Inhibitor Reactive Site," Biochemistry 28: 3628 (1991), which is hereby incorporated by reference.

4 G. Dewald, et al., "Polymorphism of Human Complement Component C6: An Amino Acid Substitution (glu/ala) Within the Second Thrombospondin Repeat Differentiates Between the Two Common Allotypes C6 A and C6 B," Biochem, Biophys. Res. Commun. 194: 458–64 (1993), which is hereby incorporated by reference.

5 P. A. Velden, et al., "Amino Acid Dimorphism in IL1A is Detectable by PCR Amplification," Hum. Mol. Genet. 2: 1753 (1993), which is hereby incorporated by reference.

6 R. M. Cawthon, et al., "Identification and Characterization of Transcripts From Theneurofibromatosis 1 Region: The Sequence and Genomic Structure of EV12 and Mapping of Other Transcripts," Genomics 7: 555–65 (1990), which is hereby incorporated by reference.

7 C. C. Brooks, et al., "Association of the Widespread A149P Hereditary Fructose Intolerance Mutation With Newly Identified Sequence Polymorphisms in the Aldolase B Gene," Am. J. Human Genetics 52: 835–40 (1993), which is hereby incorporated by reference.

8 W. Poller, et al., "Sequence Polymorphism in the Human Alpha-2-Macroglobulin (A2M) Gene," Nucleic Acids Res. 19: 198 (1991), which is hereby incorporated by reference.

9 T. Gloudemans, "An Ava II Restriction Fragment Length Polymorphism in the Insulin-Like Growth Factor II Gene and the Occurrence of Smooth Muscle Tumors," Cancer Res. 53: 5754-58 (1993), which is hereby incorporated by reference.

10 C. M. Diepstraten, et al., "A CCA/CCG Neutral Dimorphism in the Codon for Pro 626 of the Human Protein S Gene PSa (PROS1)," Nucleic Acids Res. 19: 5091 (1991), which is hereby incorporated by reference.

11 M. Reina, et al., "SSCP Polymorphism in the Human Hepatic Triglyceride Lipase (LIPC) Gene," Hum. Mol. Genet. 1: 453 (1992), which is hereby incorporated by reference.

12 S. Mastuura, et al., "Investigation of the Polymorphic AvaII Site by a PCR-based Assay at the Human CD18 Gene Locus,"Human Genetics 93: 721 (1994), which is hereby incorporated by reference.

13 L. Warnich, et al., "Detection of a Frequent Polymorphism in Exon 10 of the Low-Density Lipoprotein Receptor Gene,"Human Genetics 89: 362 (1992), which is hereby incorporated by reference.

Each region was well characterized and harbored a single-base variation with only two known alleles. PCR amplifications were performed using genomic DNA isolated from whole blood using the Purgene DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, Mich.) according to the manufacturer's instructions.

A volume of 25 μl of PCR buffer (10 mM Tris/HCl pH 8.3, 10 mM KCl, 4 mM $MgCl_2$, 0.4 mM each dNTP), 10–100 ng of genomic target DNA, PCR hybrid primer pairs 1–12 (2 pmol of each primer), and 1.3 units of AmpliTaq DNA polymerase Stoffel fragment (Applied Biosystems) was placed in a thin-walled MicroAmp reaction tube (Applied Biosystems). Each hybrid primer consisted of a gene-specific 3' region (16–29 bases) and a 5' region (22 bases) corresponding to one of two sets of universal (i.e. the portions which are primer-specific) primers (See FIG. **4**, where F1=Tet, F2=Fam, and F3=Het). These primers are shown in Table 11.

TABLE 11

Primary PCR Primer Sequences

Number or Name	Primer (5'-->3')	
1	F GGAGCACGCTATCCCGTTAGACAGCCAAGGGGAACCCTGAGAG	(SEQ. ID. NO. 49)
	R CGCTGCCAACTACCGCACTATGATCGTGGTCGAGGTGGTCACCATC	(SEQ. ID. NO. 50)

TABLE 11-continued

Primary PCR Primer Sequences

Number or Name	Primer (5'-->3')	
2	F CCTCGTTGCGAGGCGTATTCTGTATTTCCTCTTCTGTAAAAGGGAAGTTTGT	(SEQ. ID. NO. 51)
	R GCGACCTGACTTGCCGAAGAACATGTCCCATCTCCTCTACCTGATAC	(SEQ. ID. NO. 52)
3	F GGAGCACGCTATCCCGTTAGACTAAAGATCTGTCTTGCGTCCCAGTCA	(SEQ. ID. NO. 53)
	R CGCTGCCAACTACCGCACTATGTATCAATTTTGCAGAGCTTAGATGGAATG	(SEQ. ID. NO. 54)
4	F CCTCGTTGCGAGGCGTATTCTGTAGCACTTGTGATCATGGTTTTAGAAATC	(SEQ. ID. NO. 1)
	R GCGACCTGACTTGCCGAAGAACTATCGTATTTGATGATCCTCATAAAGTTG	(SEQ. ID. NO. 56)
5	F GGAGCACGCTATCCCGTTAGACATCAGCCACTTGGAAGGAGCAAAC	(SEQ. ID. NO. 57)
	R CGCTGCCAACTACCGCACTATGATGGACCATGGCTGAGTCTCCTTTAG	(SEQ. ID. NO. 58)
6	F CCTCGTTGCGAGGCGTATTCTGAACCAACACGGAGAAGCATTGTTTTC	(SEQ. ID. NO. 59)
	R GCGACCTGACTTGCCGAAGAACTATTAGCCTCAATCCTCATACTGACCTCTAC	(SEQ. ID. NO. 60)
7	F GGAGCACGCTATCCCGTTAGACATCTCCTAACATCTATGTACTGGATTATCTAAATG	(SEQ. ID. NO. 61)
	R CGCTGCCAACTACCGCACTATGATCTTACTCAAGTAATCACTCACCAGTGTTG	(SEQ. ID. NO. 62)
8	F CCTCGTTGCGAGGCGTATTCTGAATGAGTCAAATTGGCCTGGACTTG	(SEQ. ID. NO. 63)
	R GCGACCTGACTTGCCGAAGAACTTAATTCCCGTGAGAAGGGAGATG	(SEQ. ID. NO. 64)
9	F CCTCGTTGCGAGGCGTATTCTGAAGGATCTGGATGAAGCCATTTCTAAAC	(SEQ. ID. NO. 65)
	R GCGACCTGACTTGCCGAAGAACTTGGAAAAGGTATTATAAGCAGAGAAAAGATG	(SEQ. ID. NO. 66)
10	F GGAGCACGCTATCCCGTTAGACAGGACCGCAAAAGGCTTTCATC	(SEQ. ID. NO. 67)
	R CGCTGCCAACTACCGCACTATGTAGCACCCAGGCTGTACCCAATTAG	(SEQ. ID. NO. 68)
11	F CCTCGTTGCGAGGCGTATTCTGATCGGGCGCTGGGCTTCAC	(SEQ. ID. NO. 69)
	R GCGACCTGACTTGCCGAAGAACATCAGATGCCGCACTCCAAGAAG	(SEQ. ID. NO. 70)
12	F GGAGCACGCTATCCCGTTAGACATAAGAGCCCACGGCGTCTCTTC	(SEQ. ID. NO. 71)
	R CGCTGCCAACTACCGCACTATGTAAGAGACAGTGCCCAGGACAGAGTC	(SEQ. ID. NO. 72)
ZipALg1	F GGAGCACGCTATCCCGTTAGAC	(SEQ. ID. NO. 73)
ZipBLg2	R CGCTGCCAACTACCGCACAT G	(SEQ. ID. NO. 74)
ZipCLg3	F CCTCGT GCGAGGCGTATTCT G	(SEQ. ID. NO. 75)
ZipDLg4	R GCGACCTGACTTGCCGAAGAAC	(SEQ. ID. NO. 76)

Solid underline denotes the ZipALg1 and ZipBLg2 sequences. Dotted underline denotes the ZipCLg1 and ZipDLg2 sequences. Linker sequences are indicated in bold. F = forward, R = reverse.

Forward and reverse hybrid primers for loci 1,3,5,7,10, and 12 contained 5' end regions identical to universal primers ALg1 and BLg2 respectively. Forward and reverse primers to loci 2,4,6,8,9, and 11 contained 5' end regions identical to universal primers CLg3 and DLg4, respectively. The rationale for using a low concentration of the hybrid primers in the PCR phase was to deplete the hybrid primers during the reaction. This would theoretically allow products with low amplification efficiencies to "catch up with" those that had high amplification efficiencies. Amplification was attained by thermal cycling for 1 cycle of 96° C. for 15 sec to denature, then 15 cycles of 94° C. for 15 sec to denature and 65° C. for 60 sec to anneal and extend.

An equal volume of PCR buffer containing a high concentration of the two pairs of universal primers (25 pmol of each primer; Table 2) and 1.3 units of Amplitaq DNA polymerase Stoffel fragment was added to the Microamp reaction tube, and thermal cycling was performed for another 25 cycles with the annealing temperature lowered to 55° C. The upstream universal primers ALg1 and CLg3 were fluorescently labeled with 6-FAM and TET, respectively. All

thermal cycling was achieved with a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems).

The primary PCR process was carried out under the following conditions:

5.9 μl	H_2O
5 μl	primer pairs 1–12 (2 pmol each primer)
2.5 μl	10X Stoffel fragment
4 μl	25 mM $MgCl_2$
5 μl	2 mM dNTP stock (each)
2.5 μl	genomic DNA (10 ng)
0.13 μl	Stoffel Frag. (1.3 units)
25 μl	Total

The primary PCR cycling conditions were as follows:

96° C. 15"
94° C. 15", 65° C. 1' × 15
65° C. Hold

The secondary PCR process was carried out under the following conditions:

13.37 μl	H_2O
5 μl	zip primer pairs (25 pmol each primer)
2.5 μl	10X Stoffel fragment
4 μl	25 mM $MgCl_2$
0.13 μl	Stoffel Frag. (1.3 units)
25 μl	Total

The secondary PCR cycling conditions were as follows:

94° C. 15", 55° C. 1' × 25
4° C. Hold

The PCR products were separated on an Applied Biosystems 373 DNA sequencer. A 3 ml aliquot of PCR sample was mixed with 3 ml of formamide containing fluorescently labeled Genescan-2500 [TAMRA] size standard (Applied Biosystems). The formamide/standard solution was prepared by adding 50 ml Genescan-2500 size standard [TAMRA] to 450 ml of formamide. The sample was heated at 95° C. for 2 min, quick cooled in ice, and electrophoresed through a denaturing 8% polyacrylamide gel in an Applied Biosystems 373 DNA sequencer running Genescan version 1.2 software. The sizes of the fluorescently labeled products were automatically computed by the Genescan analysis software using the local Southern method. The electropherograms clearly showed 12 distinct products (FIG. **28**). The uniform amount of each product was attributed to the similar size of each amplicon and the use of the primary primers with portions complementary to the secondary primers, which annealed with identical affinities to the 12 amplicons without the need to carefully adjust reaction conditions. The computed sizes of the products, which ranged from 135 to 175 bp, matched exactly to their actual sizes (see Table 12).

TABLE 12

List of PCR and LDR Products

Site Number	PCR Products Label	PCR Products Size (bp)	LDR Product Label	LDR Product Size (bp)	LDR Product Variation	LDR Product Allele
1	6-FAM	143	6-FAM	49	C	A
			TET		T	B
2	TET	145	6-FAM	35	C	A
			TET		T	B
3	6-FAM	147	6-FAM	43	A	A
			TET		C	B
4	TET	149	6-FAM	51	T	A
			TET		G	B
5	6-FAM	155	HEX	56	A	A
				58	G	B
6	TET	157	6-FAM	53	A	A
			TET		G	B
7	6-FAM	159	6-FAM	47	G	A
			TET		A	B
8	TET	161	6-FAM	45	G	A
			TET		A	B
9	TET	165	6-FAM	55	A	A
			TET		G	B
10	6-FAM	167	HEX	48	T	A
				50	G	B
11	TET	173	HEX	44	C	A
				46	T	B
12	6-FAM	175	HEX	40	G	A
				42	A	B

The dual labeling approach (Table 12) made it much easier to distinguish the products on the electropherograms (FIG. 28, compare panel A with panels B and C).

Example 10

Multiplex Ligase Detection Reaction

To avoid any possibility of labeled PCR product interfering with the detection of ligation product sequences, PCR product amplified using unlabeled PCR universal primers served as the target for the LDR. The polymerase in the PCR was inactivated by either freeze-thawing or adding EDTA/proteinase K to a final concentration of 5 mM and 100 mg/ml, respectively, and heating to 37° C. for 30 min and 95° C. for 10 min.

Proteinase K digestion was carried out under the following conditions:

20 μl	PCR product
+2.5 μl	500 mg/ml proteinase K
25 μl	Total

60° C. for 60 min.
95° C. for 10 min to heat kill proteinase K.

Four microliters of PCR product was diluted in 20 μl of LDR mix containing 50 mM Tris/HCl pH 8.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM NAD+, 10 mM DTT, LDR oligonucleotide sets 1–12 (200 fmol of each oligonucleotide), and 10 units of *Thermus aquaticus* DNA ligase (Barany, F. and Gelfand, D., "Cloning, Overexpression, and Nucleotide Sequence of a Thermostable DNA Ligase-Encoding Gene," *Gene,* 109:1–11 (1991), which is hereby incorporated by reference). Each LDR oligonucleotide probe set consisted of two allele-specific oligonucleotides and a common oligonucleotide. Each pair of discriminating allele-specific oligonucleotide probes in the LDR oligonucleotide probe sets 1,2,3,4,6,7,8, and 9 were the same size, with one oligonucleotide labeled with 6-FAM and the other labeled with TET. For LDR oligonucleotide probe sets 5, 10, 11, and 12, each pair of allele-specific oligonucleotides differed by 2 bases (the larger oligonucleotide had a 5' tail that was not complementary to the target sequence), and both oligonucleotides were labeled with HEX. Thermal cycling was performed for 1 cycle of 95° C. for 2 min to denature, then 20 cycles of 95° C. for 30 sec to denature, and 65° C. for 4 min to ligate.

The LDR process was carried out as follows:

4 μl	PCR product
2 μl	LDR oligo sets 1–12 (200 fmol each oligo)
2 μl	10X T. ligase buffer
2 μl	10 mM NAD+
1 μl	200 mM DTT
9 μl	H_2O
20 μl	Total
+1 μl Taq Ligase (10 units)	

Stock of Ligase: 0.5 μl of 625 u/ml+3.2 μl 10X buffer+ 27.5 μl H_2O

LDR cycling conditions were as follows:

94° C. 2'
94° C. 30", 65° C. 4' × 20
4° C. Hold

A 3 μl aliquot of LDR sample was mixed with 3 μl of formamide containing fluorescently labeled Genescan-2500 [TAMRA] size standard (Applied Biosystems). The sample was heated at 95° C. for 2 min, quick cooled in ice, and electrophoresed through a denaturing 10% polyacrylamide gel in an Applied Biosystems 373 DNA sequencer running Genescan version 1.2 software. The sizes of the fluorescently labeled products were automatically computed by the Genescan analysis software using the local Southern method. LDR profiles of two individuals are shown in FIG. **29**. When each fluorescent dye was analyzed independently (FIG. **29**, panels B–D and F–H), it was very easy to determine the alleles present for each locus. A simple "A" or "B" code was assigned to each LDR product (Table 12) and used to score the genotypes. See Table 13 as follows:

TABLE 13

Genotypes Determined by PCR-LDR for 5 Individuals

	Individual Site Number											
	1	2	3	4	5	6	7	8	9	10	11	12
1	AB	AB	AB	AB	AB	AB	AB	AA	AA	AB	AB	AB
2	AA	BB	AB	BB	AB	AB	BB	AA	AB	AA	AA	AA
3	AB	AB	AB	BB	AA	BB	BB	AA	AA	AB	AB	AA
4	AA	AB	AA	AB	AB	AA	AB	AA	AB	BB	AB	AA
5	AA	AA	AA	AB	AB	AB	AB	AB	AA	AB	AB	BB

The first individual (FIGS. **29**A–D; Table 13, individual 1) was heterozygous at polymorphic sites 1–7, and 10–12. Heterozygosity at sites 1–4 and 6–7 was indicated by the detection of both 6-FAM and TET labeled products (FIG. **29**, panels B and C) at the respective positions on the electropherograms. Heterozygosity at sites 5 and 11 –12 was indicated by the presence of two HEX labeled products, differing in size by 2 bases, for each of these loci (FIG. **29**, panel D). In contrast, the one product detected at sites 8 and 9 (FIG. **29**, panels B and C) established that each of these loci was homozygous. The second individual (FIGS. **29**E–F and G–H; Table 13, individual 2) was heterozygous only at sites 3,5,6, and 9 and homozygous at sites 1,2,4,7,8, 10–12. There was a total of 8 differences in the genotypes at these positions between the two persons. Three additional individuals were typed, and all 5 persons had distinct genotypes based on the 12 loci (Table 13).

Although the invention has been described in detail for the purpose of illustration, it is understood that such details are solely for that purpose. The variations can be made therein by those skilled in the art without departing from the spirit of the scope of the invention which is defined by the following claims.

SEQUENCE LISTING

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<400> SEQUENCE: 2

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<210> SEQ ID NO 3
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<400> SEQUENCE: 3

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<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 6

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cacagtctct cggcgctggg caataataca tagtgcggta gttggc 46

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<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 7

cacgctatcc cgttagacat cttagtaatt gaggtgcgtg tttgtgcctg tc 52

<210> SEQ ID NO 8
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<400> SEQUENCE: 8

ctgggagaga ccggcgcaca ttactacata gtgcggtagt tggc 44

<210> SEQ ID NO 9
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<210> SEQ ID NO 12
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<223> OTHER INFORMATION: Description of Artificial Sequence: Primer

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<223> OTHER INFORMATION: Description of Artificial Sequence:
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<400> SEQUENCE: 29

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Oligonucleotide Sequence

<400> SEQUENCE: 30

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<210> SEQ ID NO 31
<211> LENGTH: 22

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<212> TYPE: DNA
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<220> FEATURE:
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Oligonucleotide Sequence

<400> SEQUENCE: 31

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Oligonucleotide Sequence

<400> SEQUENCE: 32

tcaagtccaa ggccattggc ttata 25

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acacagcagc ttactccaga ga 22

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<400> SEQUENCE: 35

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<223> OTHER INFORMATION: Description of Artificial Sequence:
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<210> SEQ ID NO 37
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<223> OTHER INFORMATION: Description of Artificial Sequence:
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<400> SEQUENCE: 41

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<400> SEQUENCE: 43

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<400> SEQUENCE: 47

gacatccagg cccccgac 18

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tactatgaca ccgtcatcag caga 24

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<223> OTHER INFORMATION: Description of Artificial Sequence: Primer Sequence

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Sequence

<400> SEQUENCE: 55

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<400> SEQUENCE: 59

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<210> SEQ ID NO 60
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<400> SEQUENCE: 61

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<400> SEQUENCE: 62

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<400> SEQUENCE: 63

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<210> SEQ ID NO 64
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<210> SEQ ID NO 71
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<400> SEQUENCE: 71

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<400> SEQUENCE: 72

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<210> SEQ ID NO 73
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<220> FEATURE:
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<400> SEQUENCE: 73

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<210> SEQ ID NO 74
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<223> OTHER INFORMATION: Description of Artificial Sequence: Primer Sequence

<400> SEQUENCE: 74

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<210> SEQ ID NO 75
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<223> OTHER INFORMATION: Description of Artificial Sequence: Primer Sequence

<400> SEQUENCE: 75

cctcgtgcga ggcgtattct g 21

<210> SEQ ID NO 76
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Primer Sequence

<400> SEQUENCE: 76

gcgacctgac ttgccgaaga ac 22

What is claimed:

1. A method for identifying one or more different target nucleotide sequences comprising:

providing a sample potentially containing one or more target nucleotide sequences comprising sequence differences;

providing one or more oligonucleotide probe sets, each set comprising (a) a first oligonucleotide probe comprising a target-specific portion and a 5' upstream primer-specific portion and (b) a second oligonucleotide probe comprising a target-specific portion and a 3' downstream primer-specific portion, wherein the first and second oligonucleotide probes in each particular set are suitable for ligation together when hybridized on a corresponding target nucleotide sequence, but have a mismatch which-interferes with such ligation when first and second oligonucleotide probes are hybridized to any other nucleotide sequence present in the sample;

providing a ligase;

blending the sample, the one or more oligonucleotide probe sets, and the ligase to form a ligase detection reaction mixture;

subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles to form a ligation product sequence comprising (a) the 5' upstream primer specific portion, (b) the target-specific portions, and (c) the 3' downstream primer-specific portion, when the respective target nucleotide sequence of the corresponding oligonucleotide probe set is present in the sample;

providing one or a plurality of oligonucleotide primer sets, each set comprising (a) an upstream primer and (b) a downstream primer;

providing a polymerase;

blending the ligase detection reaction mixture with the one or a plurality of oligonucleotide primer sets, and the polymerase to form a polymerase chain reaction mixture;

subjecting the polymerase chain reaction mixture to one or more polymerase chain reaction cycles to form extension products comprising the ligation product sequence and/or complements thereof; and

detecting the extension products to identify one or more target nucleotide sequences in the sample.

2. The method according to claim **1**, wherein the upstream primer is complementary to the 5' upstream primer-specific portion of the ligation product sequence and the downstream primer is complementary to the 3' downstream primer-specific portion of the ligation product sequence.

3. The method according to claim **1**, wherein one of the oligonucleotide probes in each set comprises a restriction site, said method further comprising:

restriction digesting each extension product at the restriction site to produce extension product fragments, wherein the restriction site is positioned in each of the oligonucleotide probe sets to produce an extension

product fragment with a unique length so that it can be distinguished from other nucleic acids in the polymerase chain reaction mixture after said restriction digesting; and

separating the extension product fragments by size or electrophoretic mobility, wherein said detecting differentiates the extension product fragments which differ in size.

4. The method according to claim **1**, wherein the ligation product sequence of the oligonucleotide probes in each particular set produces an extension product of unique length, said method further comprising:

separating the extension products by size or electrophoretic mobility, wherein said detecting differentiates the extension products which differ in size.

5. The method according to claim **1**, wherein the oligonucleotide probes in each set are configured so that the sequence of the ligation product sequence from each oligonucleotide probe set is unique and can be distinguished from other nucleic acids in the polymerase chain reaction mixture, said method further comprising:

providing a solid support with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the unique nucleotide sequences of given probe sets;

contacting the polymerase chain reaction mixture, after said subjecting it to one or more polymerase chain reaction cycles, with the solid support under conditions effective to hybridize the extension products to the capture oligonucleotides in a base-specific manner, wherein said detecting indicates the presence of extension products captured using the unique nucleotide sequence portions to identify one or more target nucleotide sequences in the sample.

6. The method according to claim **1**, wherein, in each primer set, one primer comprises a detectable reporter label and the other primer comprises an addressable array-specific portion which is linked to the 5' end of that primer and remains single stranded after said subjecting the polymerase chain reaction mixture to one or more polymerase chain reaction cycles, said method further comprising:

providing a solid support with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the addressable array-specific portions;

contacting the polymerase chain reaction mixture, after said subjecting it to one or more polymerase chain reaction cycles, with the solid support under conditions effective to hybridize the extension products to the capture oligonucleotides in a base-specific manner, wherein said detecting indicates the presence of extension products captured at particular sites to identify one or more target nucleotide sequences in the sample.

7. The method according to claim **1**, wherein the relative amounts of two or more differing sequences are present in a sample in unknown amounts with a plurality of target nucleotide sequences being quantified and a set of oligonucleotide primers being useful in amplifying all the ligation product sequences formed by the oligonucleotide probe sets in each particular probe group, the oligonucleotide probe sets forming a plurality of oligonucleotide probe groups, each group comprised of two or more of the oligonucleotide probe sets, wherein oligonucleotide probe sets in the same group comprise the same 5' upstream primer-specific portion and the same 3' downstream primer-specific portion, said method further comprising:

quantifying the relative amount of the extension products, after said detecting and

comparing relative amounts of the extension products generated to provide a quantitative measure of the relative level of the two or more target nucleotide sequences in the sample.

8. The method according to claim **7**, wherein one of the oligonucleotide probes in each set comprises a restriction site, said method further comprising:

restriction digesting the extension products at the restriction site to produce extension product fragments, wherein the restriction site is positioned in each of the oligonucleotide probe sets to produce an extension product fragment with a unique length so that it can be distinguished from other nucleic acids in the polymerase chain reaction mixture after said restriction digesting; and

separating the extension product fragments by size or electrophoretic mobility, wherein said detecting is carried out by size differences in the extension product fragments.

9. The method according to claim **7**, wherein oligonucleotide probe sets in the same group comprise the same 5' upstream primer-specific portion and the same 3' downstream primer-specific portion, and the ligation product sequences of oligonucleotide probes in each particular set have a unique length product so that they can be distinguished from other nucleic acids in the polymerase chain reaction mixture, said method further comprising:

separating the extension products by size or electrophoretic mobility, wherein said detecting is carried out by size differences in the extension products.

10. The method according to claim **7**, wherein the ligation product sequences of oligonucleotide probes in each particular set comprise unique sequences so that they can be distinguished from other nucleic acids in the polymerase chain reaction mixture, said method further comprising:

providing a solid support with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the unique nucleotide sequences of given probe sets;

contacting the polymerase chain reaction mixture, after said subjecting it to one or more polymerase chain reaction cycles, with the solid support under conditions effective to hybridize the extension products to the capture oligonucleotides in a base-specific manner; and

detecting the presence of extension products captured at particular sites.

11. The method according to claim **1**, wherein one or both oligonucleotide probes in each particular set comprise blocking groups at their non-ligating ends with blocking groups rendering the ligation product sequence of the oligonucleotide probes in a particular set substantially resistant to exonuclease digestion, said method further comprising:

subjecting the ligase detection reaction mixture to exonuclease digestion after said subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles and

inactivating the exonuclease.

12. The method according to claim **1**, wherein the ligation product sequence of the oligonucleotide probes in each particular set generates an extension product of unique length, said method further comprising:

separating the extension products by size or electrophoretic mobility, wherein said detecting differentiates the extension products which differ in size.

13. The method according to claim **12**, wherein the one or more target nucleotide sequences are present in the sample in unknown amounts with a plurality of target nucleotide sequences being quantified, said method further comprising:

providing a known amount of one or more marker target nucleotide sequences;

providing one or a plurality of oligonucleotide probe groups, each group comprised of two or more of the oligonucleotide probe sets, comprising probe sets specifically designed for the marker target nucleotide sequences, wherein one or both oligonucleotide probes in each particular set are blocked at their non-ligating ends, wherein oligonucleotide probe sets in the same group comprise either (a) the same 5' upstream primer-specific portion or (b) the same 3' downstream primer-specific portion, or (c) both the same 5' upstream primer-specific portion and the same 3' downstream primer-specific portion, said ligase detection reaction mixture further comprising the marker target nucleotide sequences and the probe sets including probe sets specifically designed for the marker target nucleotide sequences, said method further comprising:

providing one or a plurality of oligonucleotide primer groups, each group comprised of two or more oligonucleotide primer sets, wherein the oligonucleotide primer sets in each group comprise either the same 5' upstream primer or the same 3' downstream primer or both the same 5' upstream primer and the same 3' downstream primer, a group of oligonucleotide primers being useful to amplify all ligation product sequences in a given group;

quantifying the amount of extension products after said detecting; and

comparing the amounts of extension products generated from the unknown sample with the amount of extension products generated from known amounts of marker target nucleotide sequences to provide a quantitative measure of the level of one or more target nucleotide sequences in the sample.

14. The method according to claim **11**, wherein one primer comprises a detectable reporter label and the other primer comprises an addressable array-specific portion which is linked to the 5' end of that primer and remains single stranded after said subjecting the polymerase chain reaction mixture to one or more polymerase chain reaction cycles, said method further comprising:

providing a solid support with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the addressable array-specific portions and

contacting the polymerase chain reaction mixture, after said subjecting it to one or more polymerase chain reaction cycles, with the solid support under conditions effective to hybridize the extension products to the capture oligonucleotides in a base-specific manner, wherein said detecting indicates the presence of extension products captured at particular sites to identify one or more target nucleotide sequences in the sample.

15. The method according to claim **14**, wherein the one or more different target sequences are present in the sample in unknown amounts with a plurality of target nucleotide sequences being quantified, one primer comprising a detectable reporter label and the other primer comprising an addressable array-specific portion which is linked to the 5' end of that primer and remains single stranded after said subjecting the polymerase chain reaction mixture to one or more polymerase chain reaction cycles, and the oligonucleotide primer sets in the same group comprise either the same 5' upstream primer or the same 3' downstream primer, wherein a group of oligonucleotide primers may be used to amplify all ligation product sequences in a given group, said method further comprising:

providing a known amount of one or more marker target nucleotide sequences;

providing one or a plurality of oligonucleotide probe groups, each group comprised of two or more of the oligonucleotide probe sets, including probe sets specifically designed for the marker target nucleotide sequences, wherein one or both oligonucleotide probe sets in the same group comprise either the same 5' upstream primer-specific portion or the same 3' downstream primer-specific portion;

providing one or a plurality of oligonucleotide primer groups, each group comprised of two or more oligonucleotide primer sets, wherein the oligonucleotide primer sets in the same group comprise either the same 5' upstream primer or the same 3' downstream primer and a group of oligonucleotide primers are used to amplify all ligation product sequences in a group;

blending the marker target nucleotide sequences and probe sets specifically designed for the marker target nucleotide sequences with the ligase detection reaction mixture;

quantifying the amount of extension products; and

comparing the amounts of extension products generated from the unknown sample with the amount of extension products generated from known amounts of marker target nucleotide sequences to provide a quantitative measure of the level of one or more target nucleotide sequences in the sample.

16. The method according to claim **1**, wherein each cycle of the ligase detection reaction is from about 30 seconds to about five minutes long.

17. The method according to claim **1**, wherein said subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles is repeated for 2 to 50 cycles.

18. The method according to claim **1**, wherein total time for said subjecting the ligase detection reaction mixture to one or more ligase detection reaction cycles is 1 to 250 minutes.

19. The method according to claim **1**, wherein the ligase is selected from the group consisting of *Thermus aquaticus* ligase, *Thermus thermophilus* ligase, *E. coli* ligase, T4 ligase, and *Pyrococcus* ligase.

20. The method according to claim **1**, wherein the target-specific portions of the oligonucleotide probes each have a hybridization temperature of 50–85° C.

21. The method according to claim **1**, wherein the target-specific portions of the oligonucleotide probes are 20 to 28 nucleotides long.

22. The method according to claim **1**, wherein the oligonucleotide probe sets are selected from the group consisting of ribonucleotides, deoxyribonucleotides, modified ribonucleotides, modified deoxyribonucleotides, modified phosphate-sugar backbone oligonucleotides, nucleotide analogues, and mixtures thereof.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

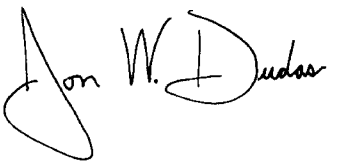
PATENT NO. : 7,166,434 B2
APPLICATION NO. : 10/843720
DATED : January 23, 2007
INVENTOR(S) : Barany et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 1 at lines 16-18, delete "This invention was developed with government funding under National lnstitutes of Health Grant No. GM41337-06. The U.S. Government may retain certain rights." and insert --This invention was made with government support under grant GM41337-06 awarded by the National lnstitutes of Health. The government has certain rights in the invention-- in its place.

Signed and Sealed this

Nineteenth Day of August, 2008



JON W. DUDAS
Director of the United States Patent and Trademark Office

Exhibit D

US007312039B2

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

(10) **Patent No.: US 7,312,039 B2**
(45) **Date of Patent: *Dec. 25, 2007**

(54) **DETECTION OF NUCLEIC ACID SEQUENCE DIFFERENCES USING COUPLED LIGASE DETECTION AND POLYMERASE CHAIN REACTIONS**

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

This patent is subject to a terminal disclaimer.

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(22) Filed: **Oct. 31, 2006**

(65) **Prior Publication Data**

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Related U.S. Application Data

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(51) **Int. Cl.**
C12Q 1/68 (2006.01)
C12P 19/34 (2006.01)

(52) **U.S. Cl.** **435/6**; 435/91.2

(58) **Field of Classification Search** 435/6, 435/91.1, 91.2
See application file for complete search history.

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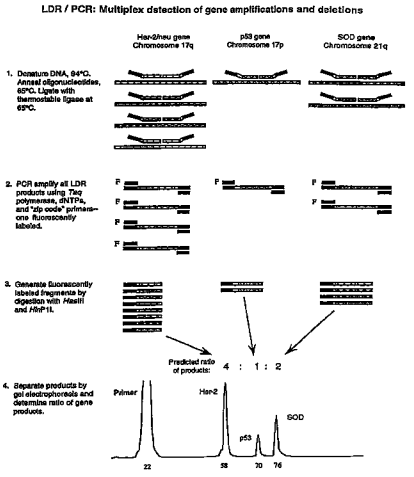
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(57) **ABSTRACT**

The present invention relates to a method for identifying a target nucleotide sequence. This method involves forming a ligation product on a target nucleotide sequence in a ligase detection reaction mixture, amplifying the ligation product to form an amplified ligation product in a polymerase chain reaction (PCR) mixture, detecting the amplified ligation product, and identifying the target nucleotide sequence. Such coupling of the ligase detection reaction and the polymerase chain reaction permits multiplex detection of nucleic acid sequence differences.

5 Claims, 29 Drawing Sheets



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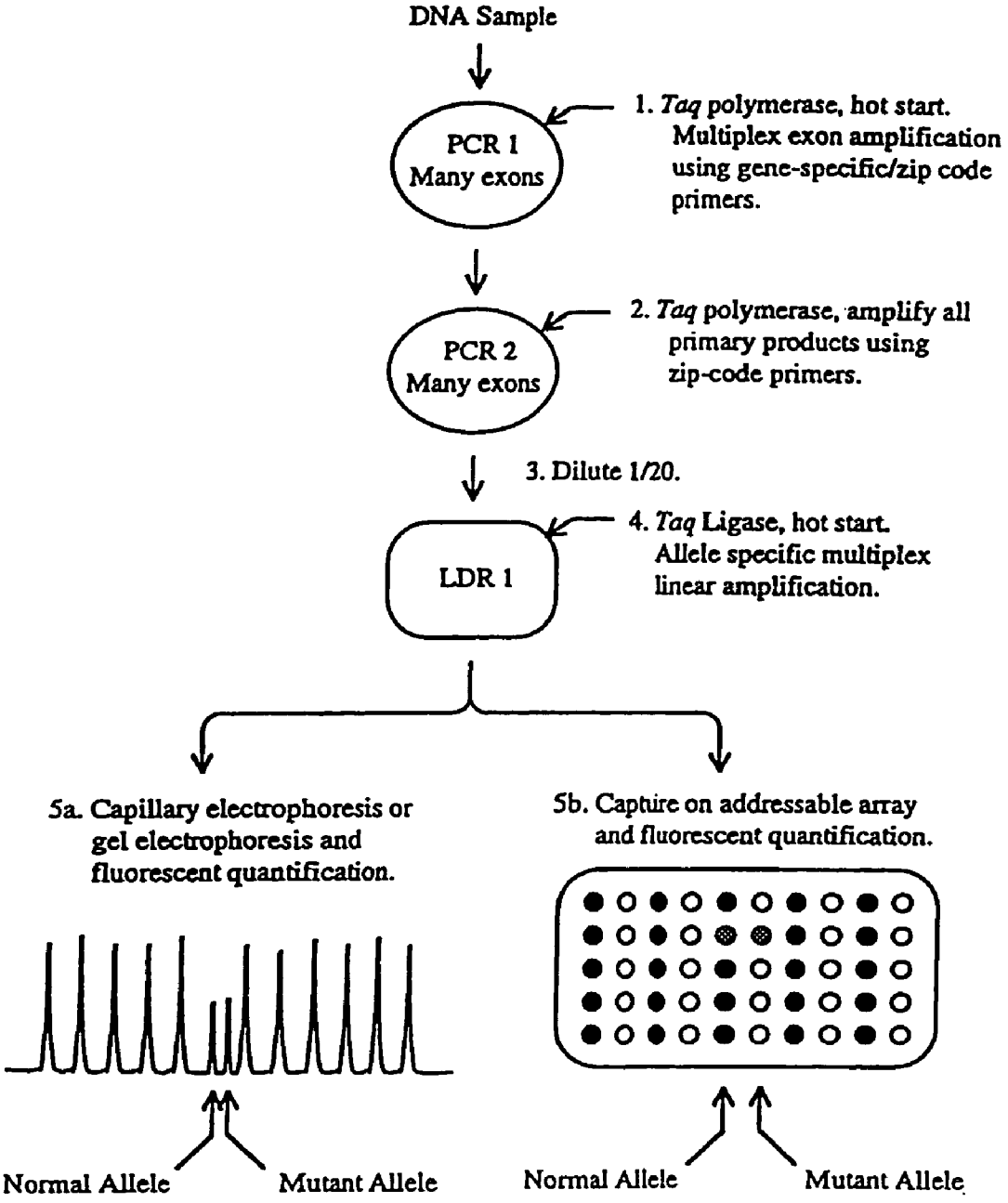

DNA Sample
PCR 1
Many exons
1. Taq polymerase, hot start. Multiplex exon amplification using gene-specific/zip code primers.
PCR 2
Many exons
2. Taq polymerase, amplify all primary products using zip-code primers.
3. Dilute 1/20.
LDR 1
4. Taq Ligase, hot start. Allele specific multiplex linear amplification.
5a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
5b. Capture on addressable array and fluorescent quantification.
Normal Allele
Mutant Allele
Normal Allele
Mutant Allele

FIG. 1

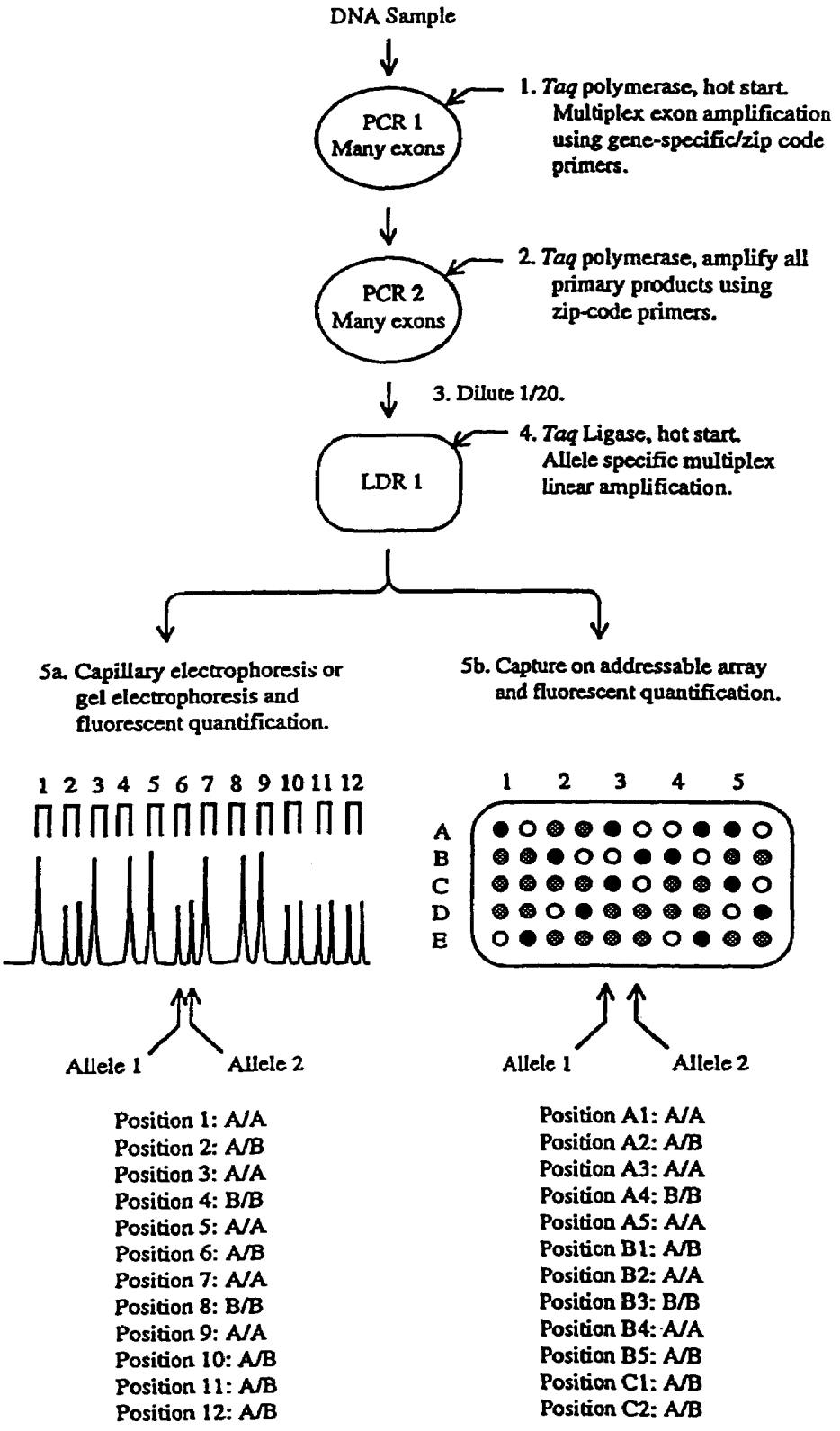

DNA Sample
PCR 1
Many exons
1. Taq polymerase, hot start. Multiplex exon amplification using gene-specific/zip code primers.
PCR 2
Many exons
2. Taq polymerase, amplify all primary products using zip-code primers.
3. Dilute 1/20.
LDR 1
4. Taq Ligase, hot start. Allele specific multiplex linear amplification.
5a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
5b. Capture on addressable array and fluorescent quantification.
1 2 3 4 5 6 7 8 9 10 11 12
Allele 1
Allele 2
Position 1: A/A
Position 2: A/B
Position 3: A/A
Position 4: B/B
Position 5: A/A
Position 6: A/B
Position 7: A/A
Position 8: B/B
Position 9: A/A
Position 10: A/B
Position 11: A/B
Position 12: A/B
1 2 3 4 5
A
B
C
D
E
Allele 1
Allele 2
Position A1: A/A
Position A2: A/B
Position A3: A/A
Position A4: B/B
Position A5: A/A
Position B1: A/B
Position B2: A/A
Position B3: B/B
Position B4: A/A
Position B5: A/B
Position C1: A/B
Position C2: A/B

FIG. 2

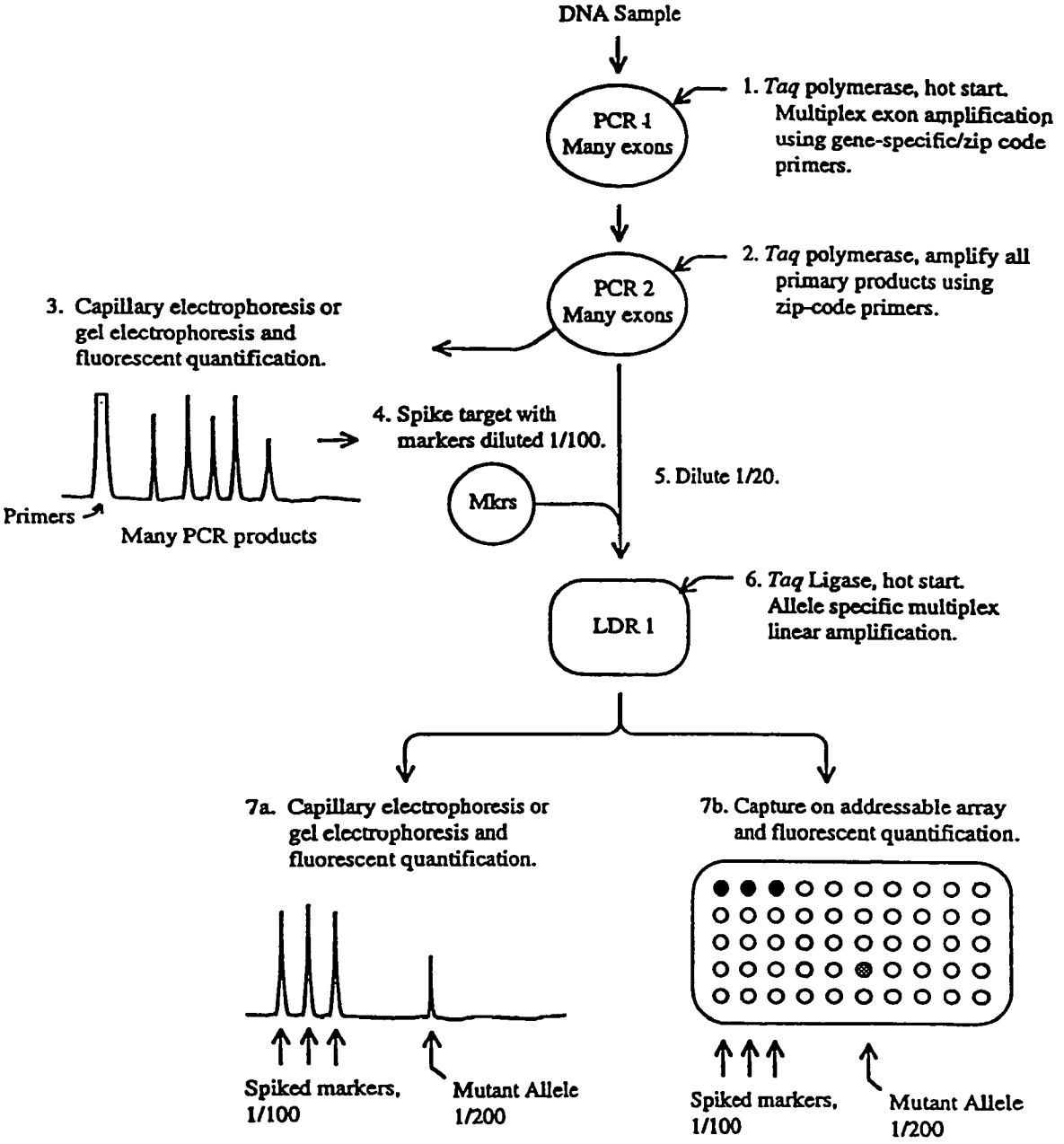

DNA Sample
PCR 1
Many exons
1. Taq polymerase, hot start. Multiplex exon amplification using gene-specific/zip code primers.
PCR 2
Many exons
2. Taq polymerase, amplify all primary products using zip-code primers.
3. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Primers
Many PCR products
4. Spike target with markers diluted 1/100.
Mkrs
5. Dilute 1/20.
LDR 1
6. Taq Ligase, hot start. Allele specific multiplex linear amplification.
7a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200
7b. Capture on addressable array and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200

FIG. 3

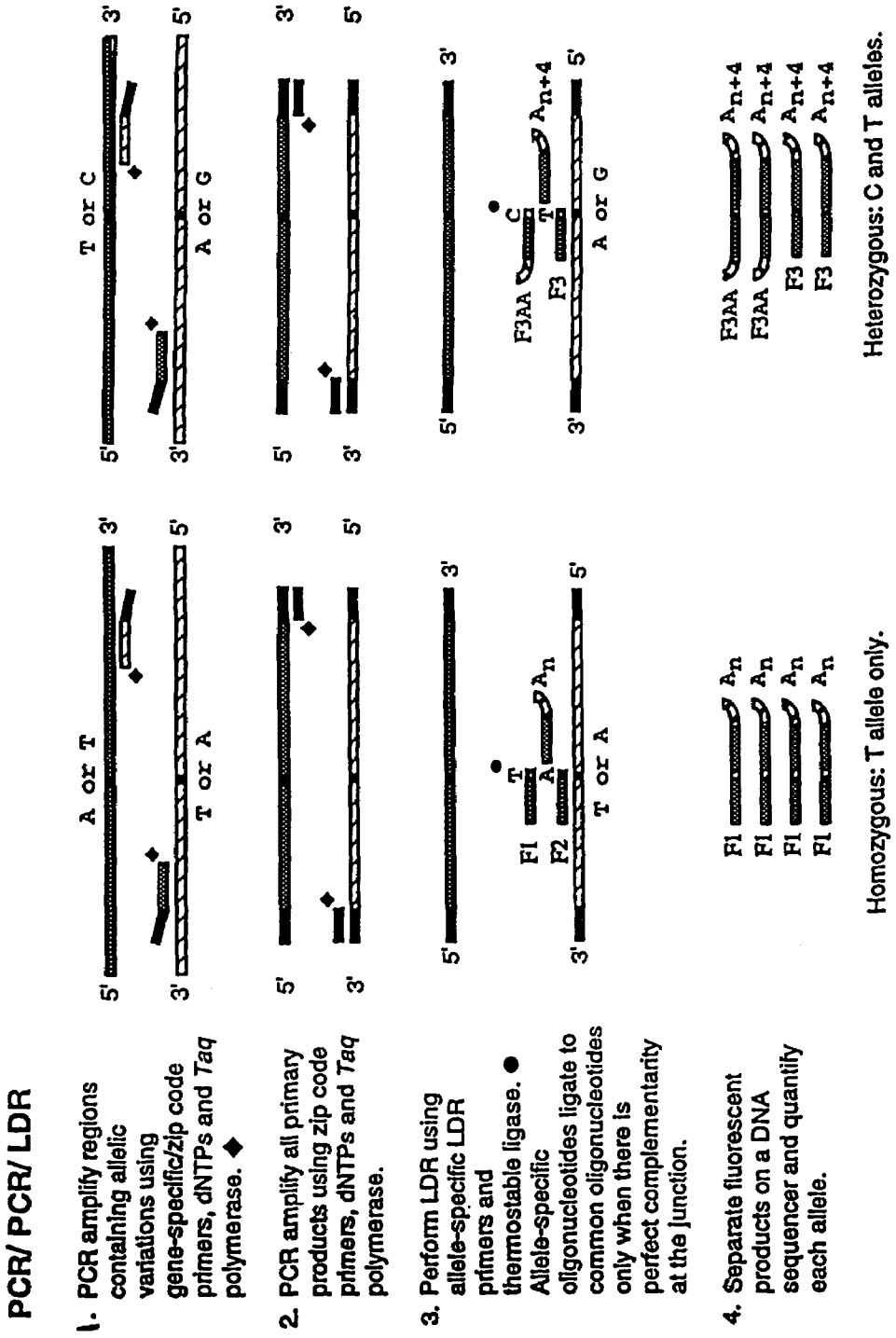

PCR/ PCR/ LDR
1. PCR amplify regions containing allelic variations using gene-specific/zip code primers, dNTPs and Taq polymerase. ◆
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase.
3. Perform LDR using allele-specific LDR primers and thermostable ligase. ● Allele-specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
4. Separate fluorescent products on a DNA sequencer and quantify each allele.
A or T
T or A
T or C
A or G
F1
F2
F3
F3AA
Homozygous: T allele only.
Heterozygous: C and T alleles.

FIG. 4

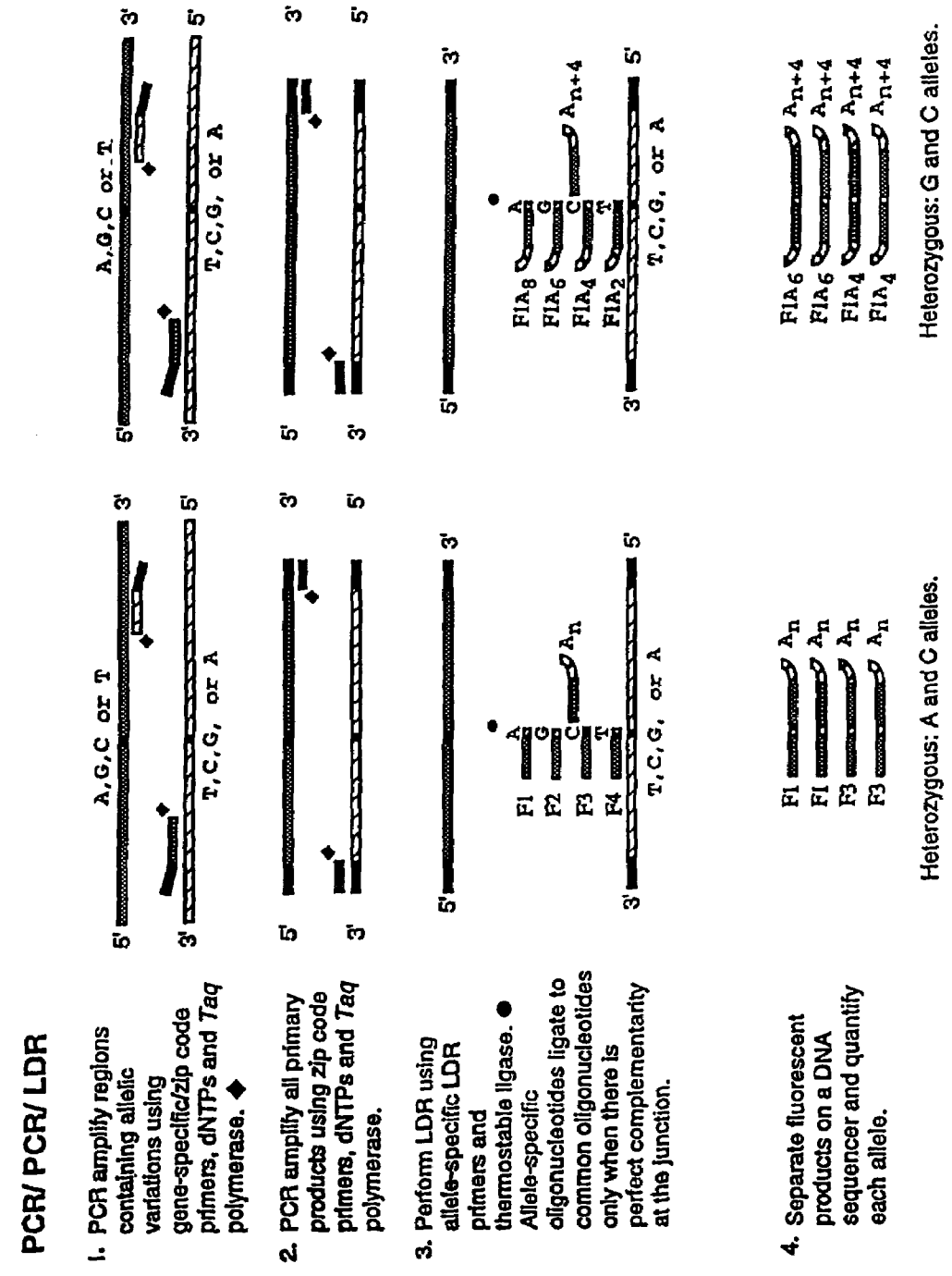

PCR/ PCR/ LDR
1. PCR amplify regions containing allelic variations using gene-specific/zip code primers, dNTPs and Taq polymerase. ◆
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase.
3. Perform LDR using allele-specific LDR primers and thermostable ligase. ● Allele-specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
4. Separate fluorescent products on a DNA sequencer and quantify each allele.
A,G,C or T
T,C,G, or A
5'
3'
F1
F2
F3
F4
A
G
C
T
An
An+4
F1A8
F1A6
F1A4
F1A2
F1A6
F1A4
F1A4
F1A4
Heterozygous: A and C alleles.
Heterozygous: G and C alleles.

FIG. 5

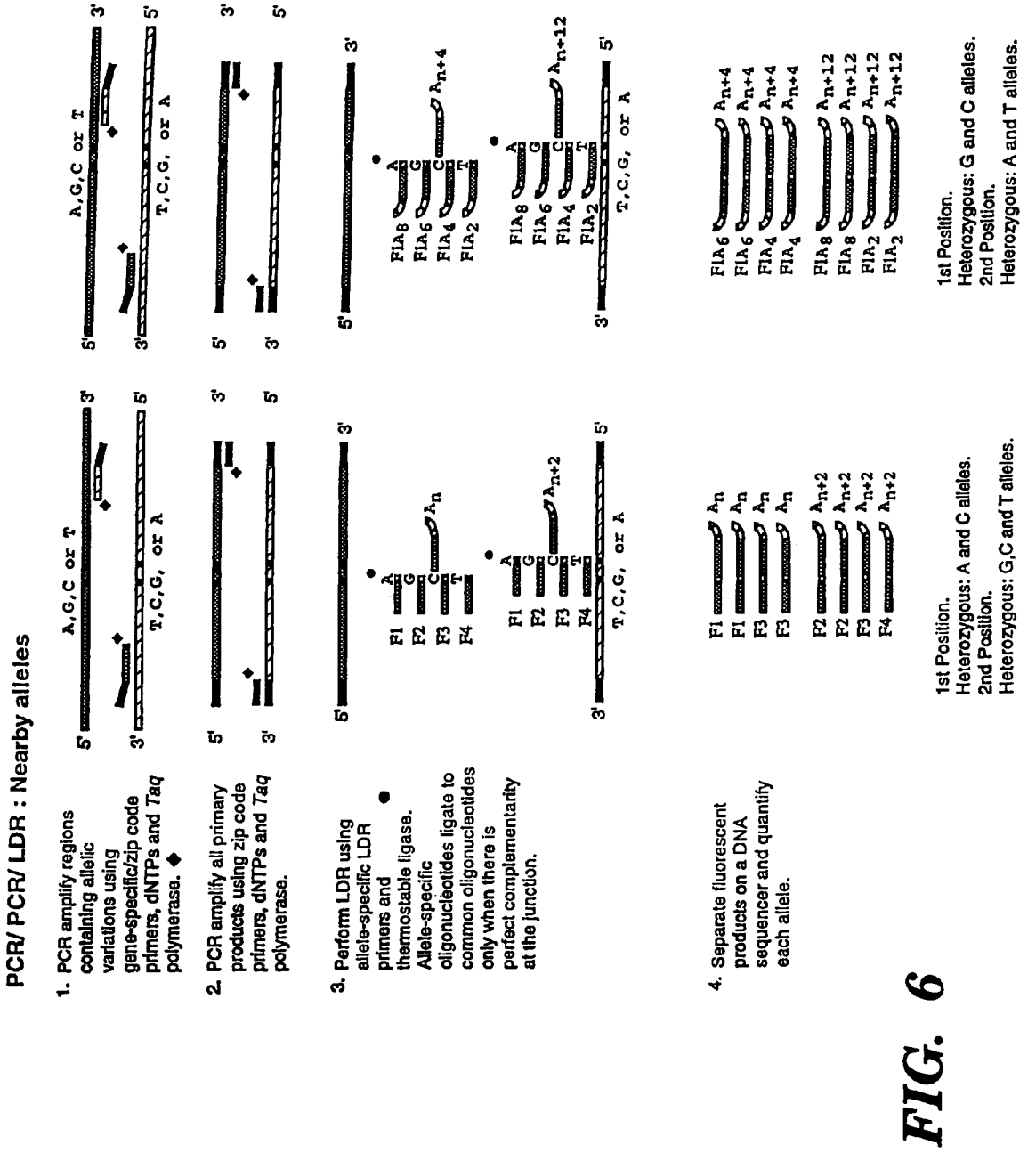

PCR/ PCR/ LDR : Nearby alleles
1. PCR amplify regions containing allelic variations using gene-specific/zip code primers, dNTPs and Taq polymerase. ◆
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase.
3. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele-specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction. ●
4. Separate fluorescent products on a DNA sequencer and quantify each allele.
A,G,C or T
T,C,G, or A
F1
F2
F3
F4
1st Position.
Heterozygous: A and C alleles.
2nd Position.
Heterozygous: G,C and T alleles.
1st Position.
Heterozygous: G and C alleles.
2nd Position.
Heterozygous: A and T alleles.

FIG. 6

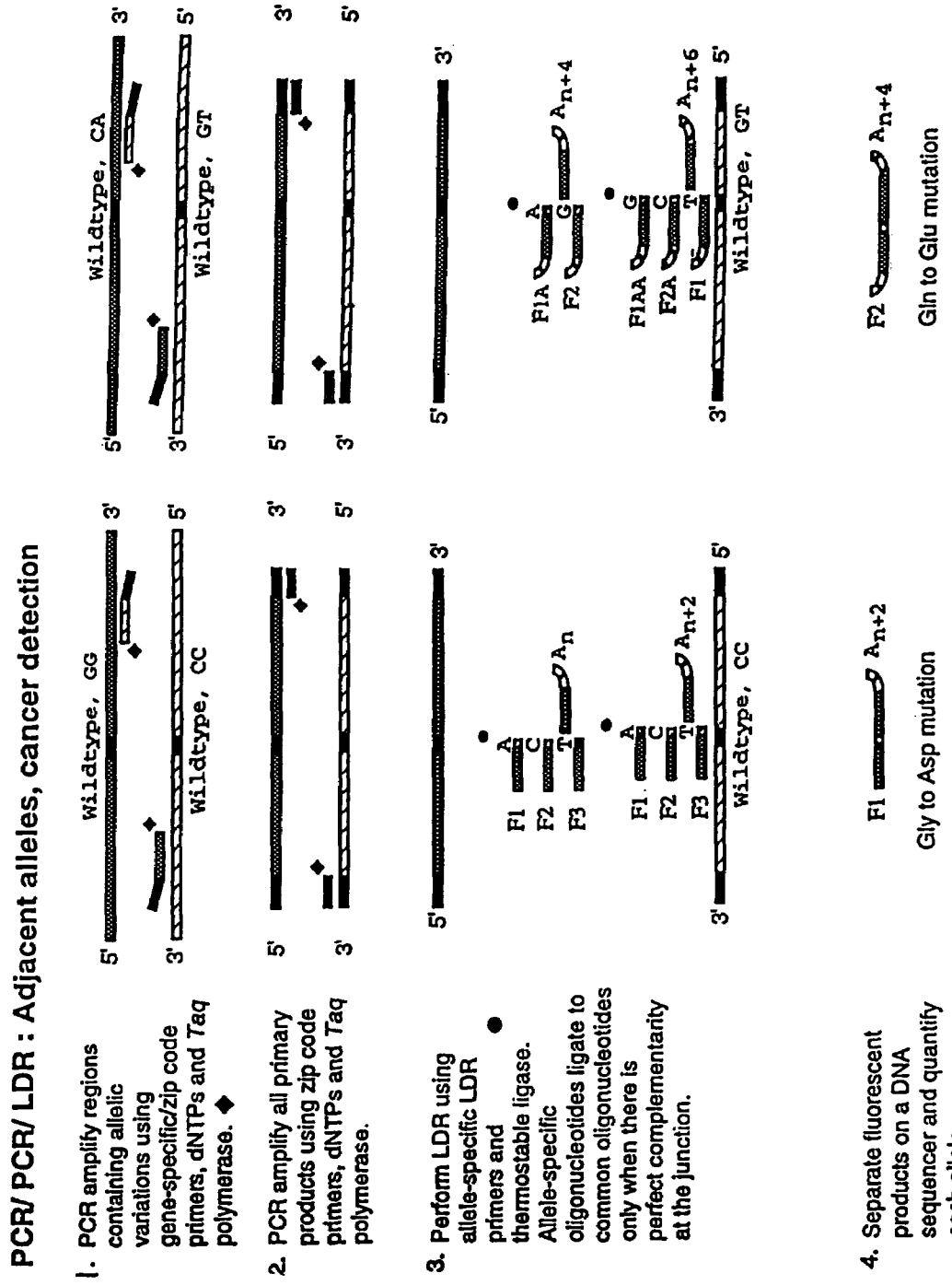

PCR/ PCR/ LDR : Adjacent alleles, cancer detection
1. PCR amplify regions containing allelic variations using gene-specific/zip code primers, dNTPs and Taq polymerase. ◆
Wildtype, GG
Wildtype, CC
Wildtype, CA
Wildtype, GT
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase.
3. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele-specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction. ●
F1
F2
F3
F1A
F2A
F1AA
4. Separate fluorescent products on a DNA sequencer and quantify each allele.
Gly to Asp mutation
Gln to Glu mutation

FIG. 7

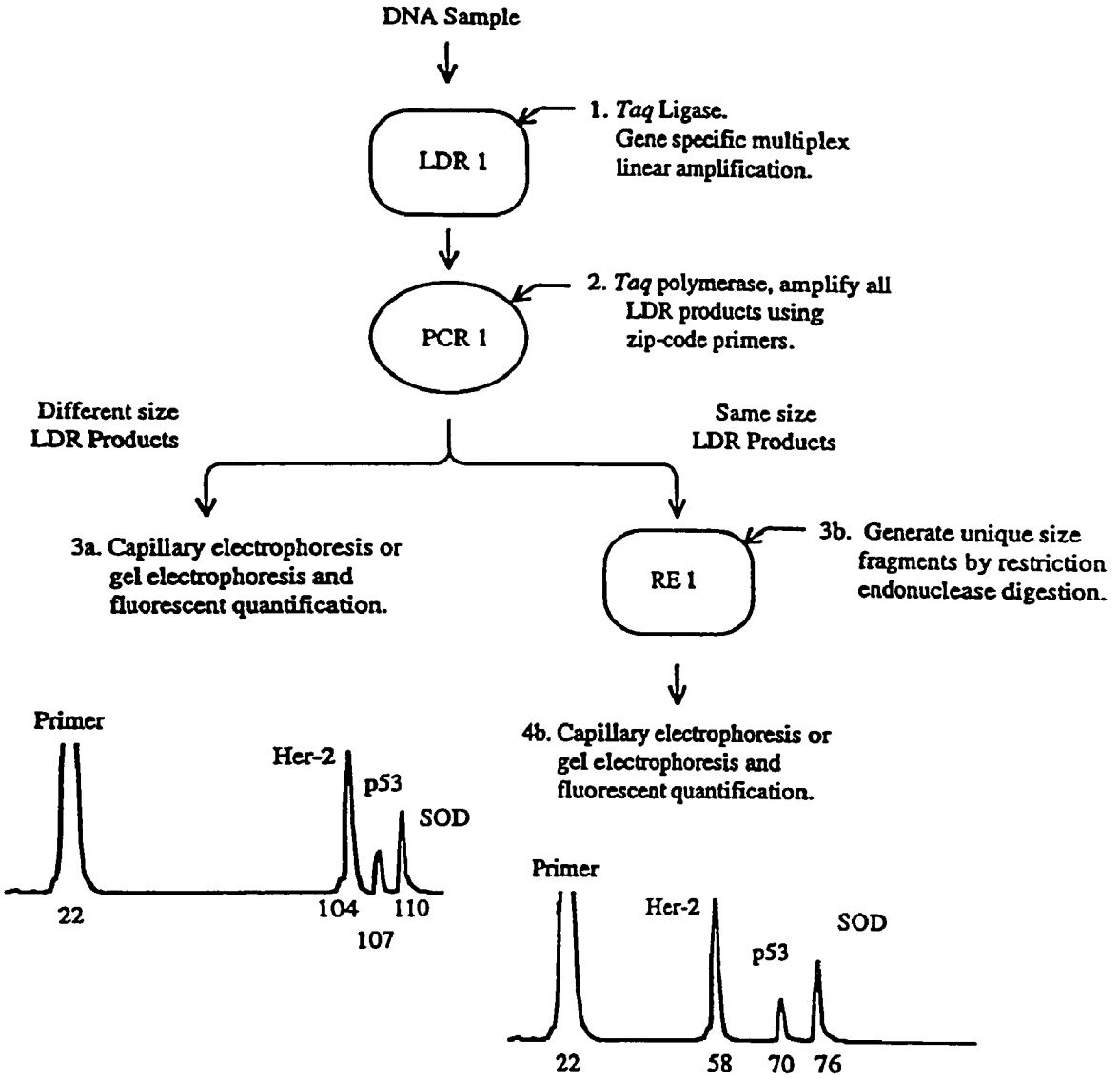

DNA Sample
LDR 1
1. Taq Ligase. Gene specific multiplex linear amplification.
PCR 1
2. Taq polymerase, amplify all LDR products using zip-code primers.
Different size LDR Products
Same size LDR Products
3a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
RE 1
3b. Generate unique size fragments by restriction endonuclease digestion.
Primer
Her-2
p53
SOD
22
104
110
107
4b. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Primer
Her-2
p53
SOD
22
58
70
76

FIG. 8

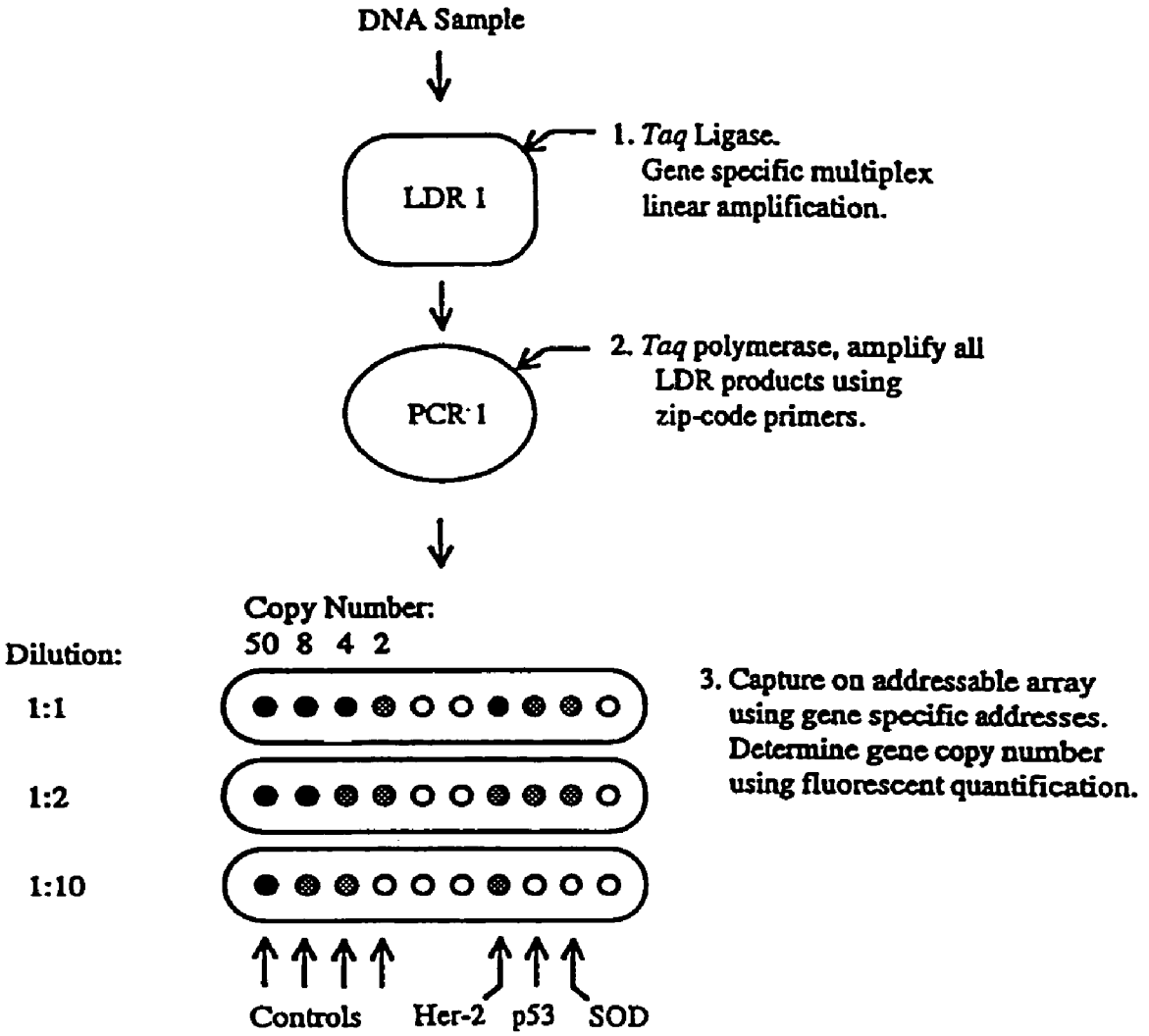

DNA Sample
LDR 1
1. Taq Ligase. Gene specific multiplex linear amplification.
PCR 1
2. Taq polymerase, amplify all LDR products using zip-code primers.
Copy Number:
50 8 4 2
Dilution:
1:1
1:2
1:10
3. Capture on addressable array using gene specific addresses. Determine gene copy number using fluorescent quantification.
Controls
Her-2
p53
SOD

FIG. 9

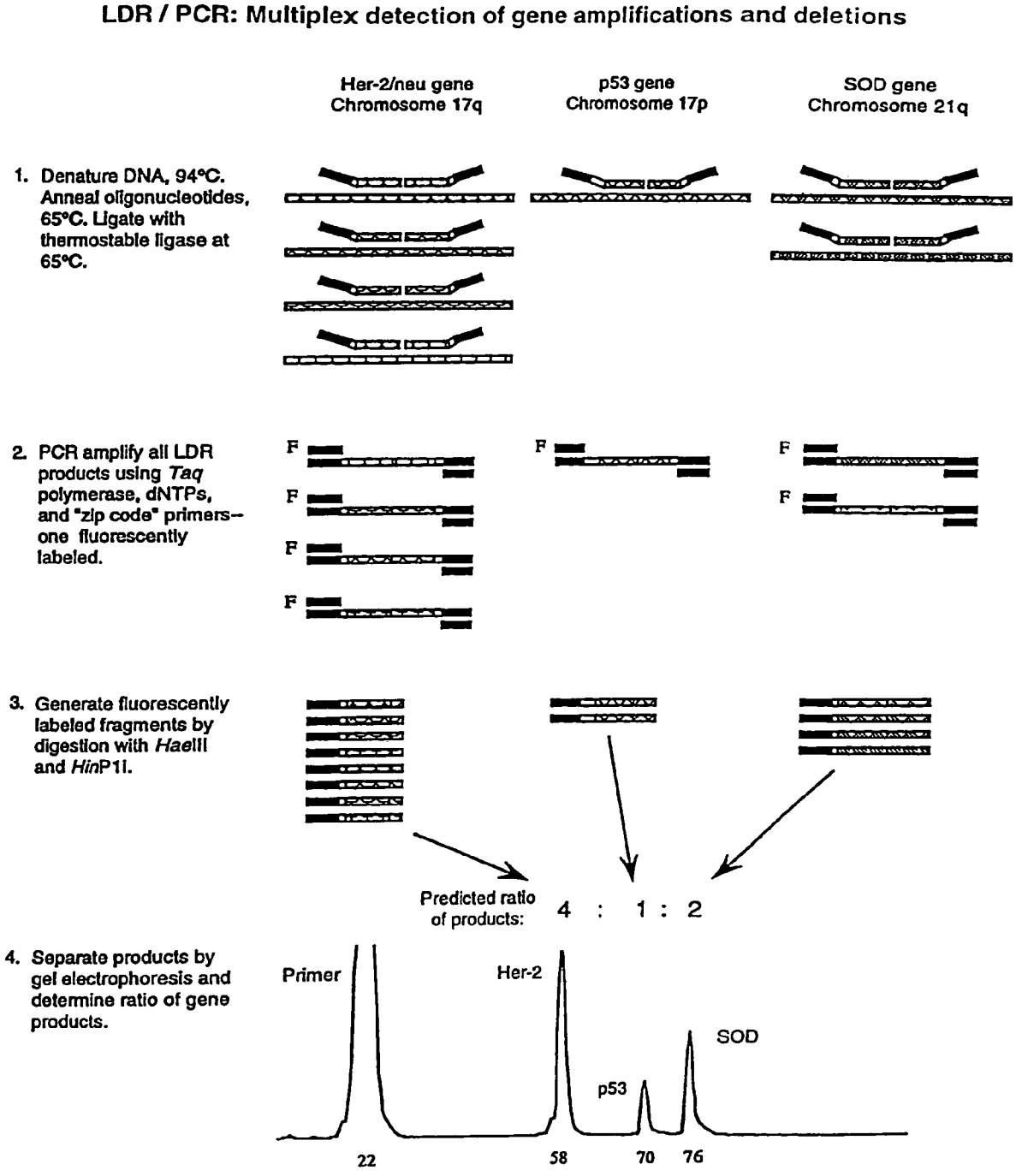

LDR / PCR: Multiplex detection of gene amplifications and deletions
Her-2/neu gene Chromosome 17q
p53 gene Chromosome 17p
SOD gene Chromosome 21q
1. Denature DNA, 94°C. Anneal oligonucleotides, 65°C. Ligate with thermostable ligase at 65°C.
2. PCR amplify all LDR products using Taq polymerase, dNTPs, and "zip code" primers--one fluorescently labeled.
F
3. Generate fluorescently labeled fragments by digestion with HaeIII and HinP1I.
Predicted ratio of products:
4 : 1 : 2
4. Separate products by gel electrophoresis and determine ratio of gene products.
Primer
Her-2
p53
SOD
22
58
70
76

FIG. 10

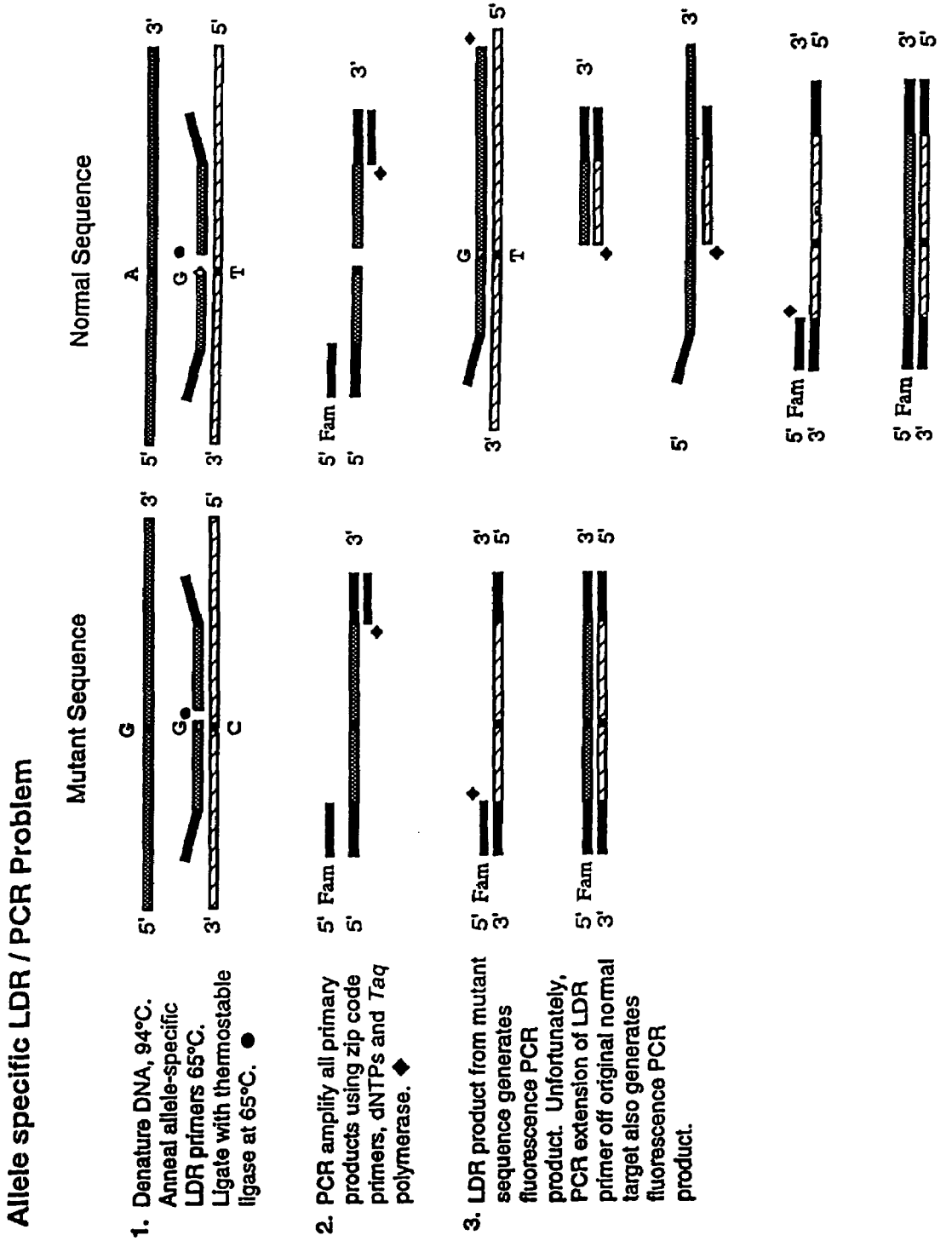

Allele specific LDR / PCR Problem
Mutant Sequence
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific LDR primers 65°C. Ligate with thermostable ligase at 65°C. ●
2. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
3. LDR product from mutant sequence generates fluorescence PCR product. Unfortunately, PCR extension of LDR primer off original normal target also generates fluorescence PCR product.
5' Fam
3'
5'

FIG. 11

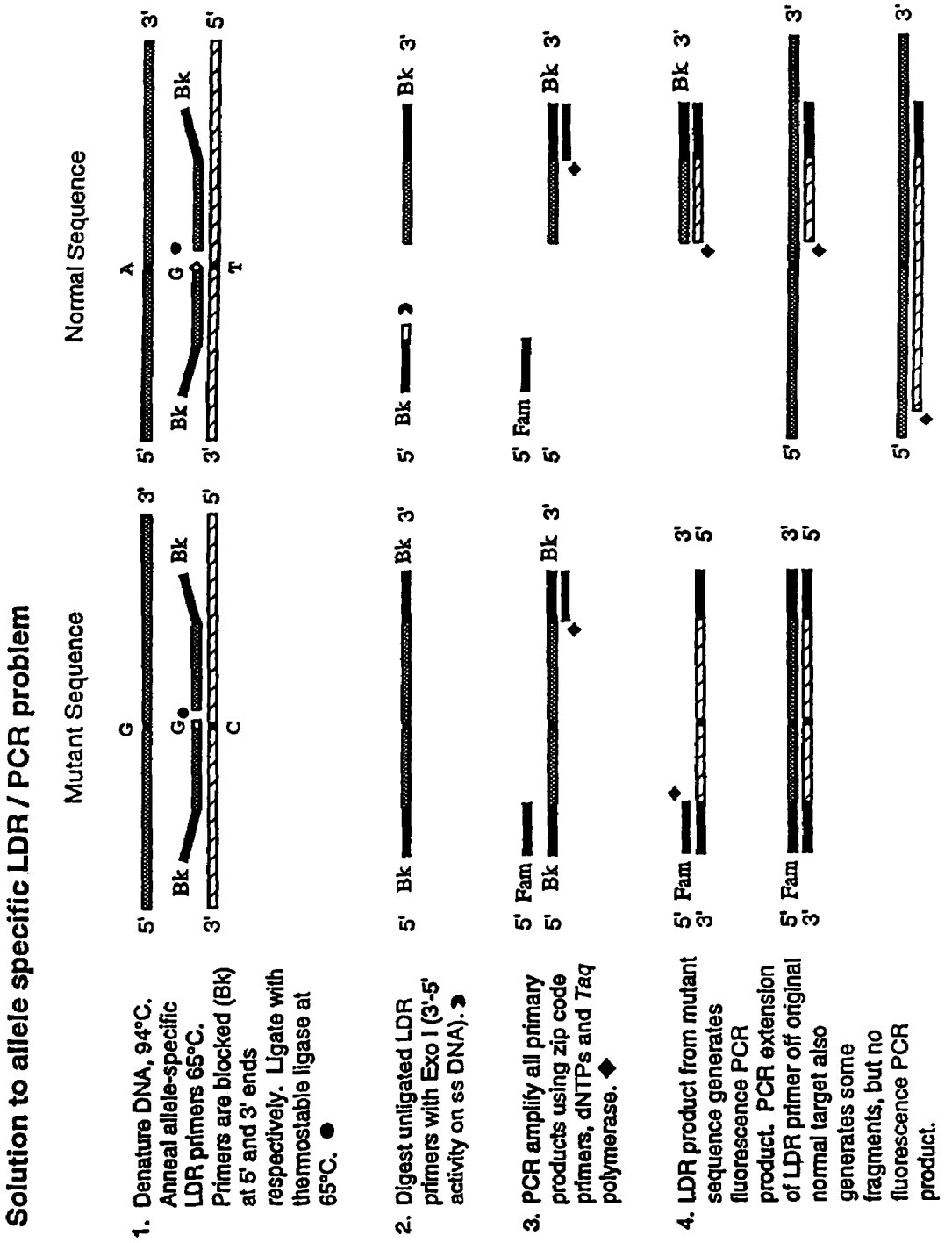

Solution to allele specific LDR / PCR problem
Mutant Sequence
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific LDR primers 65°C. Primers are blocked (Bk) at 5' and 3' ends respectively. Ligate with thermostable ligase at 65°C. ●
2. Digest unligated LDR primers with Exo I (3'-5' activity on ss DNA). ◗
3. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
4. LDR product from mutant sequence generates fluorescence PCR product. PCR extension of LDR primer off original normal target also generates some fragments, but no fluorescence PCR product.
5' 3' Bk Fam G A T C

FIG. 12

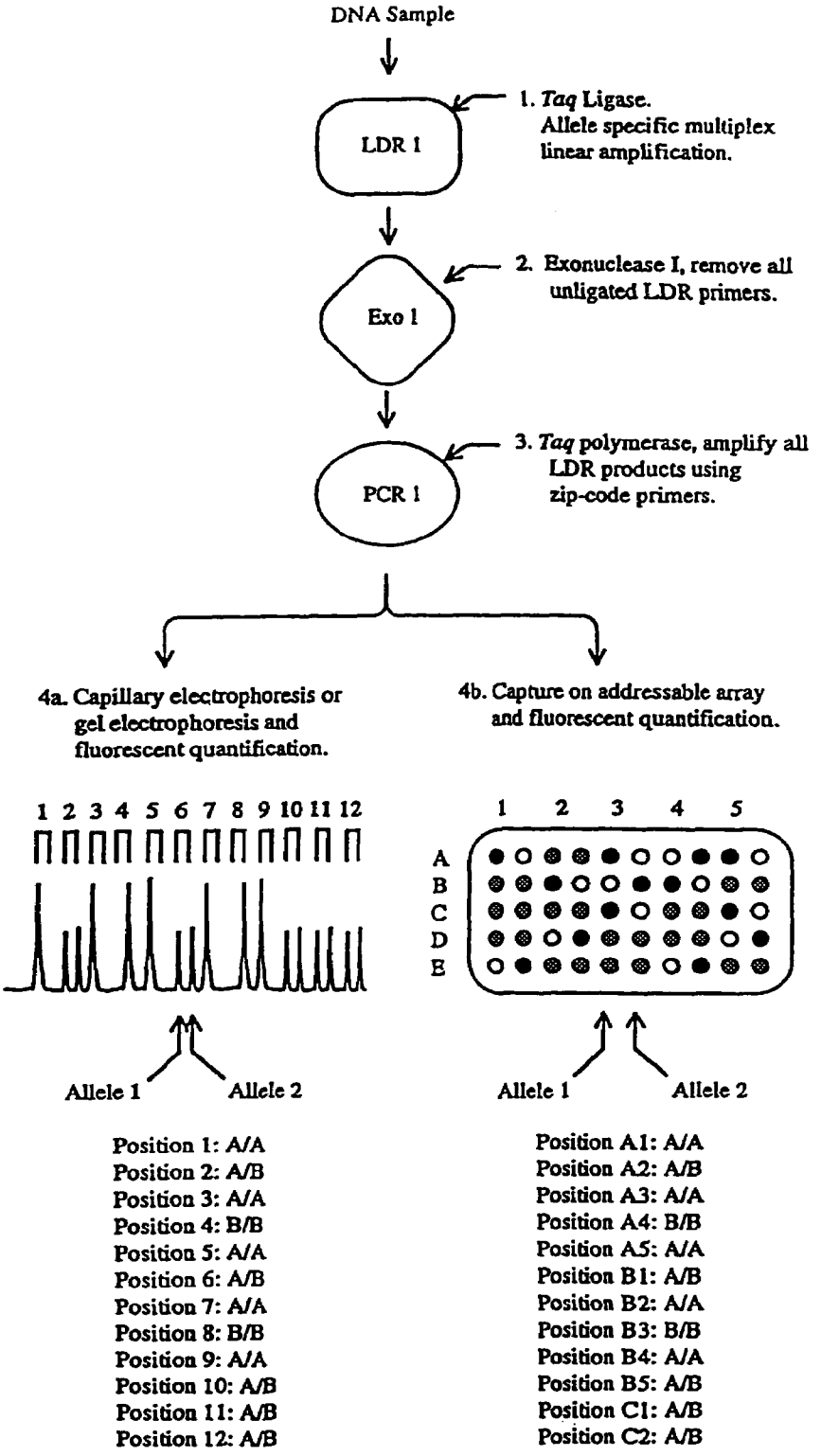

DNA Sample
LDR 1
1. Taq Ligase. Allele specific multiplex linear amplification.
Exo 1
2. Exonuclease I, remove all unligated LDR primers.
PCR 1
3. Taq polymerase, amplify all LDR products using zip-code primers.
4a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
4b. Capture on addressable array and fluorescent quantification.
1 2 3 4 5 6 7 8 9 10 11 12
Allele 1
Allele 2
Position 1: A/A
Position 2: A/B
Position 3: A/A
Position 4: B/B
Position 5: A/A
Position 6: A/B
Position 7: A/A
Position 8: B/B
Position 9: A/A
Position 10: A/B
Position 11: A/B
Position 12: A/B
1 2 3 4 5
A
B
C
D
E
Allele 1
Allele 2
Position A1: A/A
Position A2: A/B
Position A3: A/A
Position A4: B/B
Position A5: A/A
Position B1: A/B
Position B2: A/A
Position B3: B/B
Position B4: A/A
Position B5: A/B
Position C1: A/B
Position C2: A/B

FIG. 13

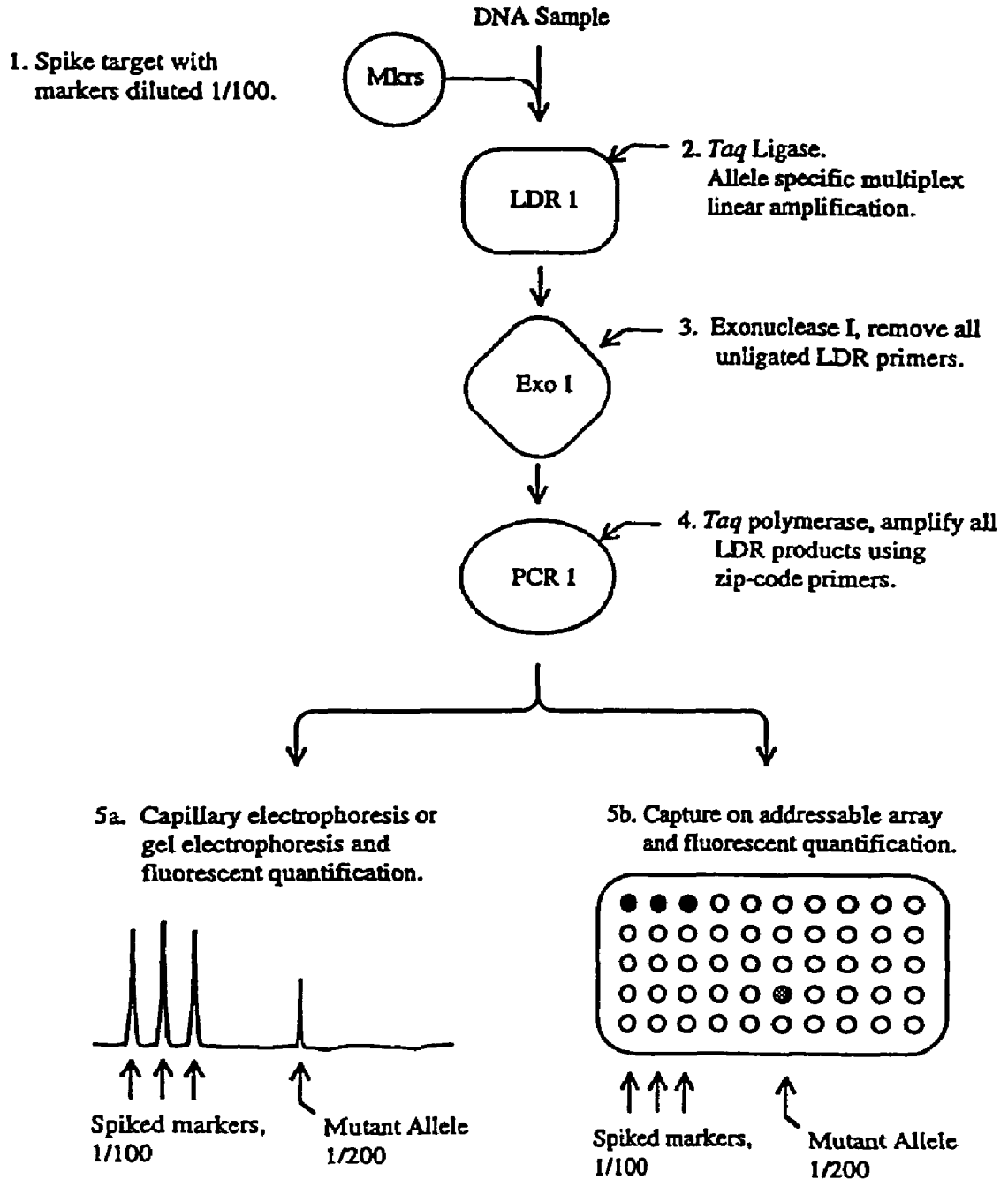

DNA Sample
1. Spike target with markers diluted 1/100.
Mkrs
LDR 1
2. Taq Ligase. Allele specific multiplex linear amplification.
Exo I
3. Exonuclease I, remove all unligated LDR primers.
PCR 1
4. Taq polymerase, amplify all LDR products using zip-code primers.
5a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200
5b. Capture on addressable array and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200

FIG. 14

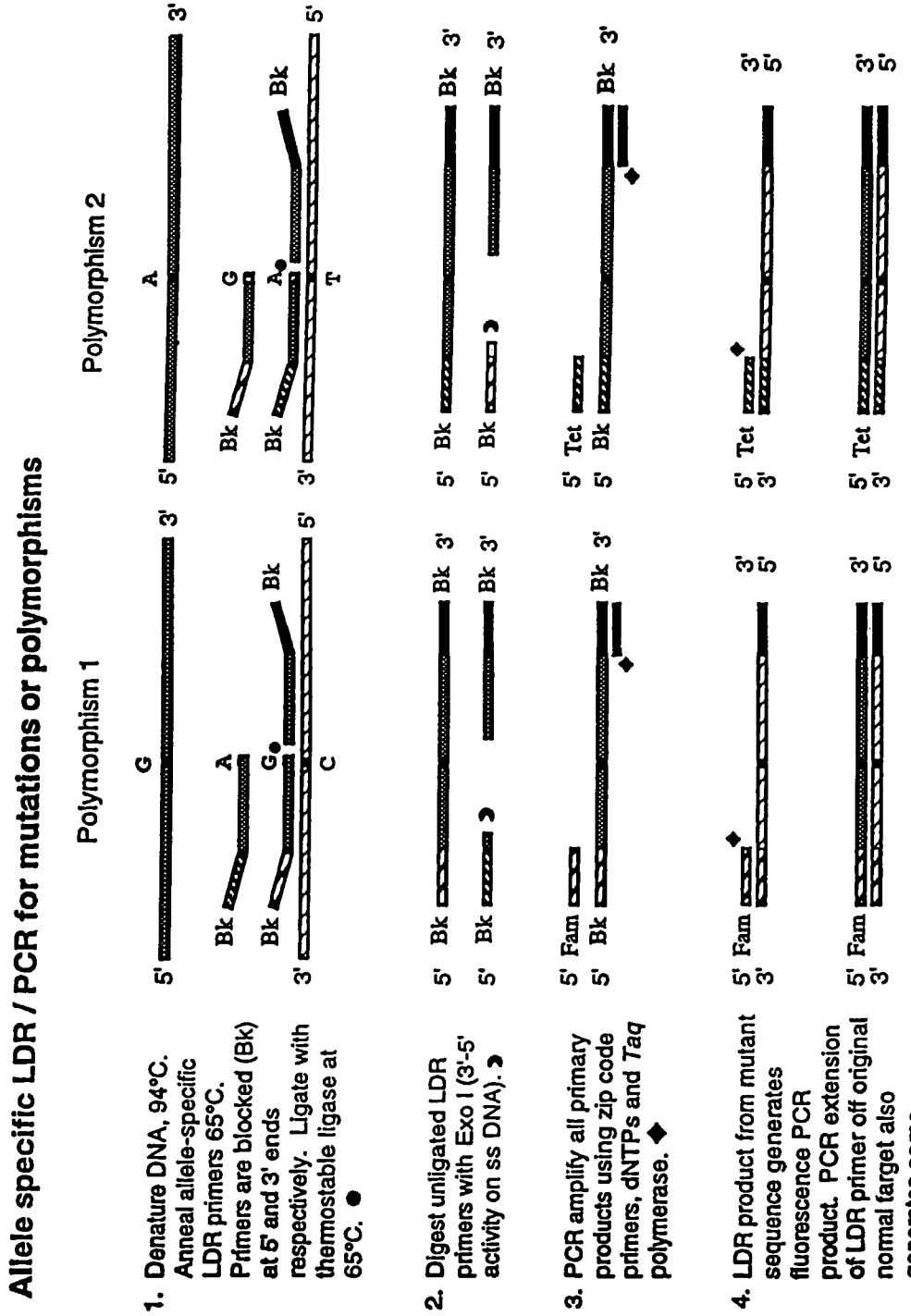

Allele specific LDR / PCR for mutations or polymorphisms
Polymorphism 1
Polymorphism 2
1. Denature DNA, 94°C. Anneal allele-specific LDR primers 65°C. Primers are blocked (Bk) at 5' and 3' ends respectively. Ligate with thermostable ligase at 65°C. ●
2. Digest unligated LDR primers with Exo I (3'-5' activity on ss DNA). ➤
3. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
4. LDR product from mutant sequence generates fluorescence PCR product. PCR extension of LDR primer off original normal target also generates some fragments, but no fluorescence PCR product.
5' 3' G A C T Bk Fam Tet

FIG. 15

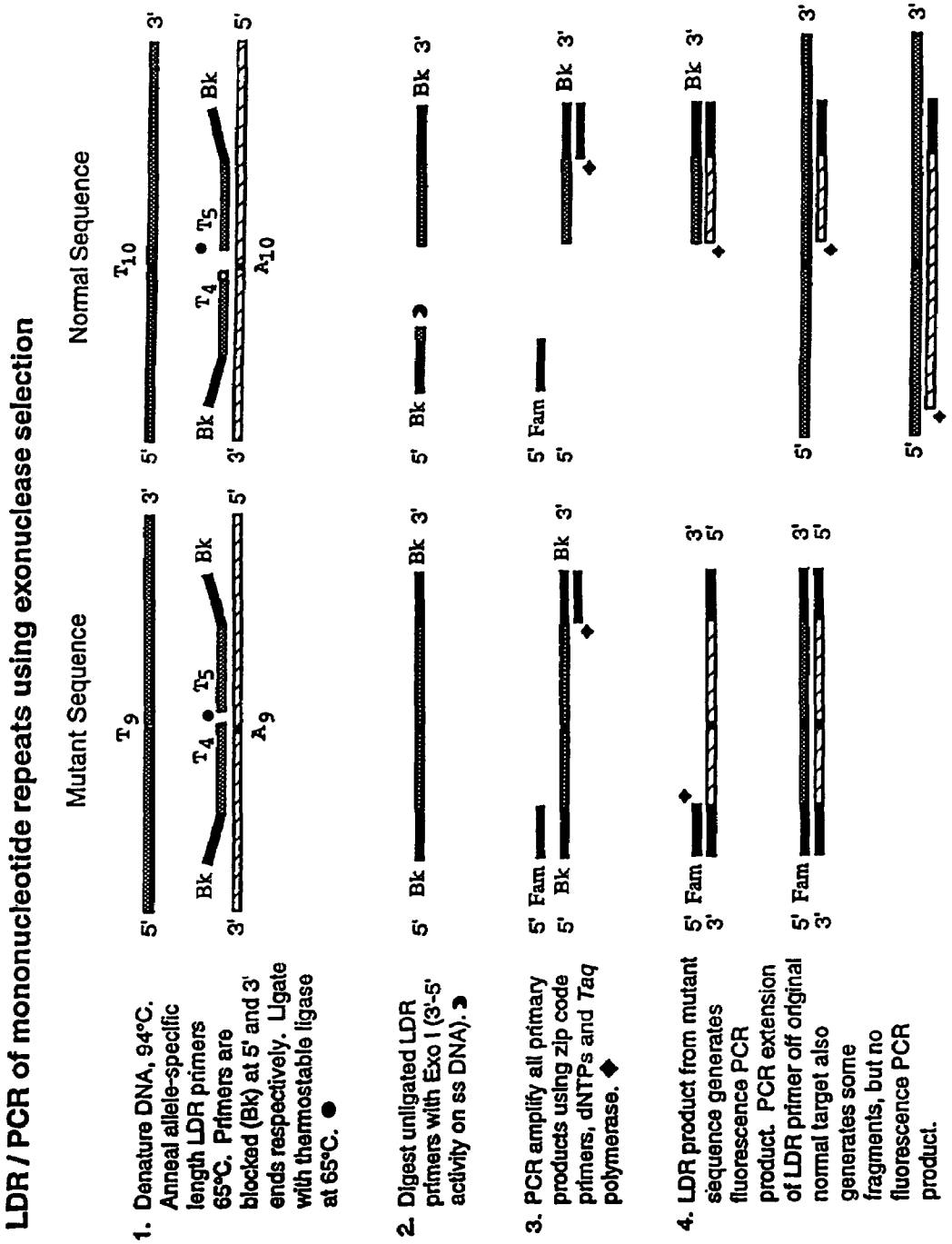

LDR / PCR of mononucleotide repeats using exonuclease selection
Mutant Sequence
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific length LDR primers 65°C. Primers are blocked (Bk) at 5' and 3' ends respectively. Ligate with thermostable ligase at 65°C. ●
2. Digest unligated LDR primers with Exo I (3'-5' activity on ss DNA). ➲
3. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
4. LDR product from mutant sequence generates fluorescence PCR product. PCR extension of LDR primer off original normal target also generates some fragments, but no fluorescence PCR product.
T9
T10
T4
T5
A9
A10
Bk
Fam
5'
3'

FIG. 16

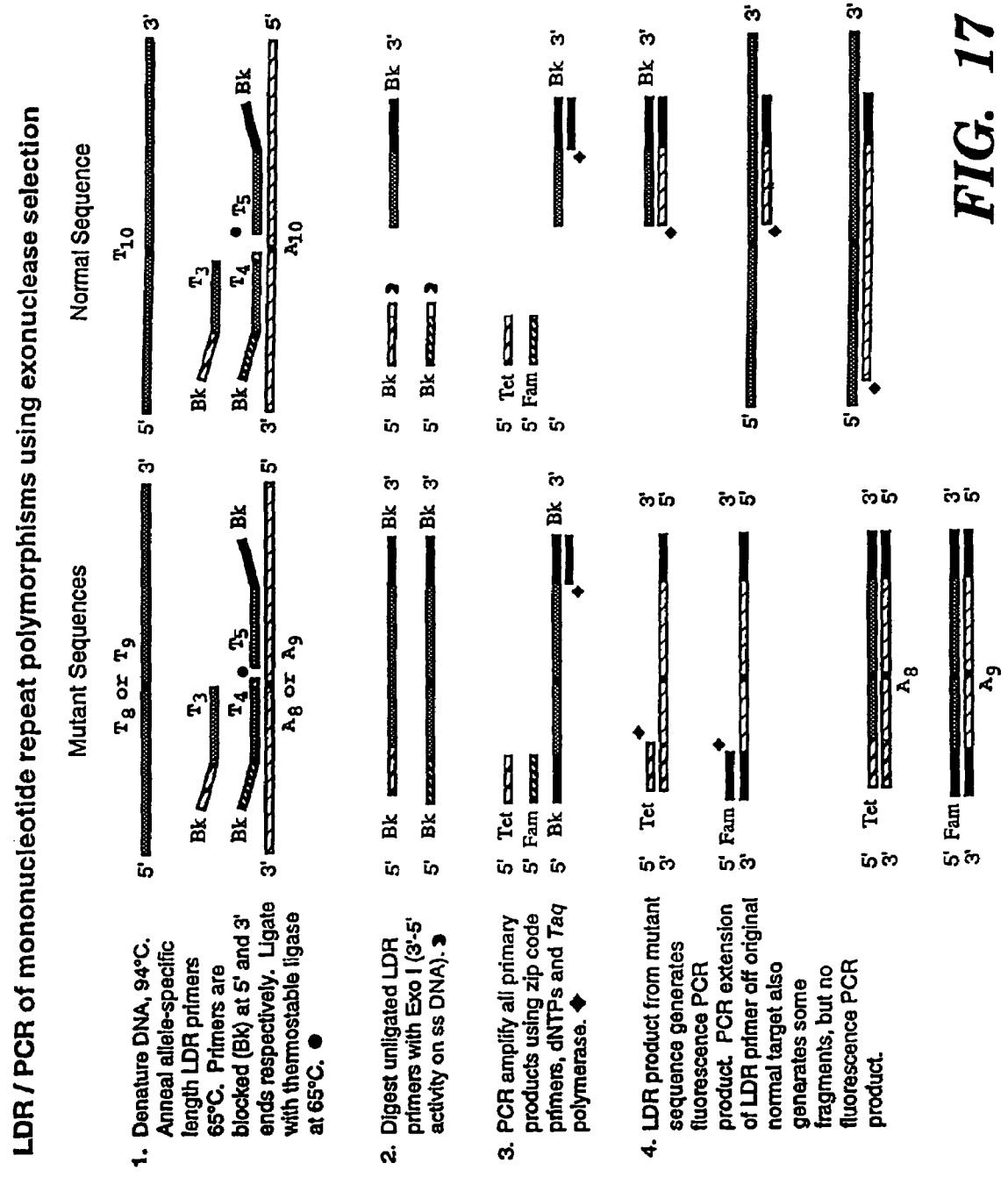

LDR / PCR of mononucleotide repeat polymorphisms using exonuclease selection
Mutant Sequences
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific length LDR primers 65°C. Primers are blocked (Bk) at 5' and 3' ends respectively. Ligate with thermostable ligase at 65°C. ●
2. Digest unligated LDR primers with Exo I (3'-5' activity on ss DNA). ➽
3. PCR amplify all primary products using zip code primers, dNTPs and Taq polymerase. ◆
4. LDR product from mutant sequence generates fluorescence PCR product. PCR extension of LDR primer off original normal target also generates some fragments, but no fluorescence PCR product.
T8 or T9
A8 or A9
T10
A10
T3
T4
T5
Bk
Tet
Fam
A8
A9

FIG. 17

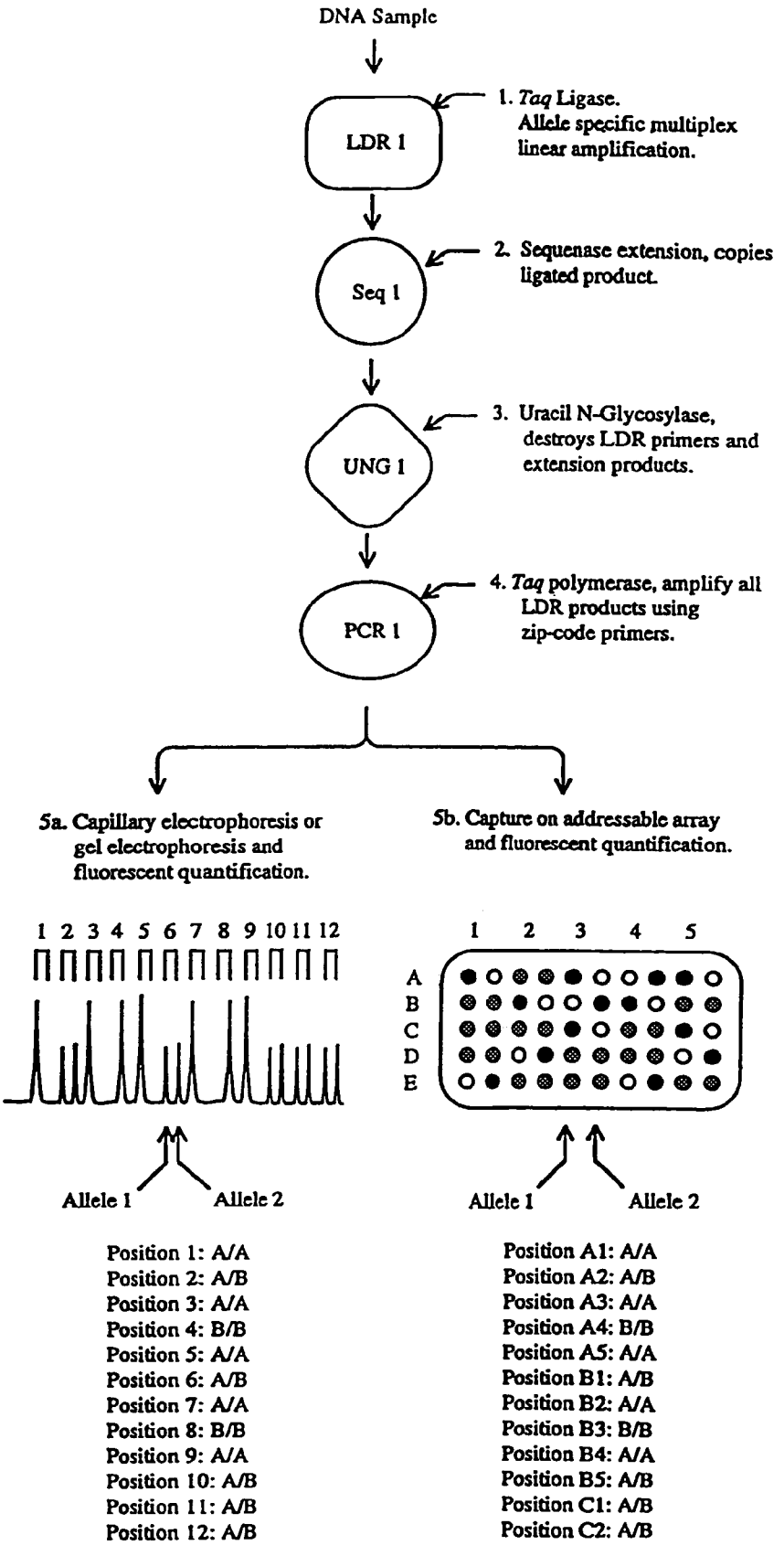

DNA Sample
LDR 1
1. Taq Ligase. Allele specific multiplex linear amplification.
Seq 1
2. Sequenase extension, copies ligated product.
UNG 1
3. Uracil N-Glycosylase, destroys LDR primers and extension products.
PCR 1
4. Taq polymerase, amplify all LDR products using zip-code primers.
5a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
5b. Capture on addressable array and fluorescent quantification.
1 2 3 4 5 6 7 8 9 10 11 12
1 2 3 4 5
A
B
C
D
E
Allele 1
Allele 2
Allele 1
Allele 2
Position 1: A/A
Position 2: A/B
Position 3: A/A
Position 4: B/B
Position 5: A/A
Position 6: A/B
Position 7: A/A
Position 8: B/B
Position 9: A/A
Position 10: A/B
Position 11: A/B
Position 12: A/B
Position A1: A/A
Position A2: A/B
Position A3: A/A
Position A4: B/B
Position A5: A/A
Position B1: A/B
Position B2: A/A
Position B3: B/B
Position B4: A/A
Position B5: A/B
Position C1: A/B
Position C2: A/B

FIG. 18

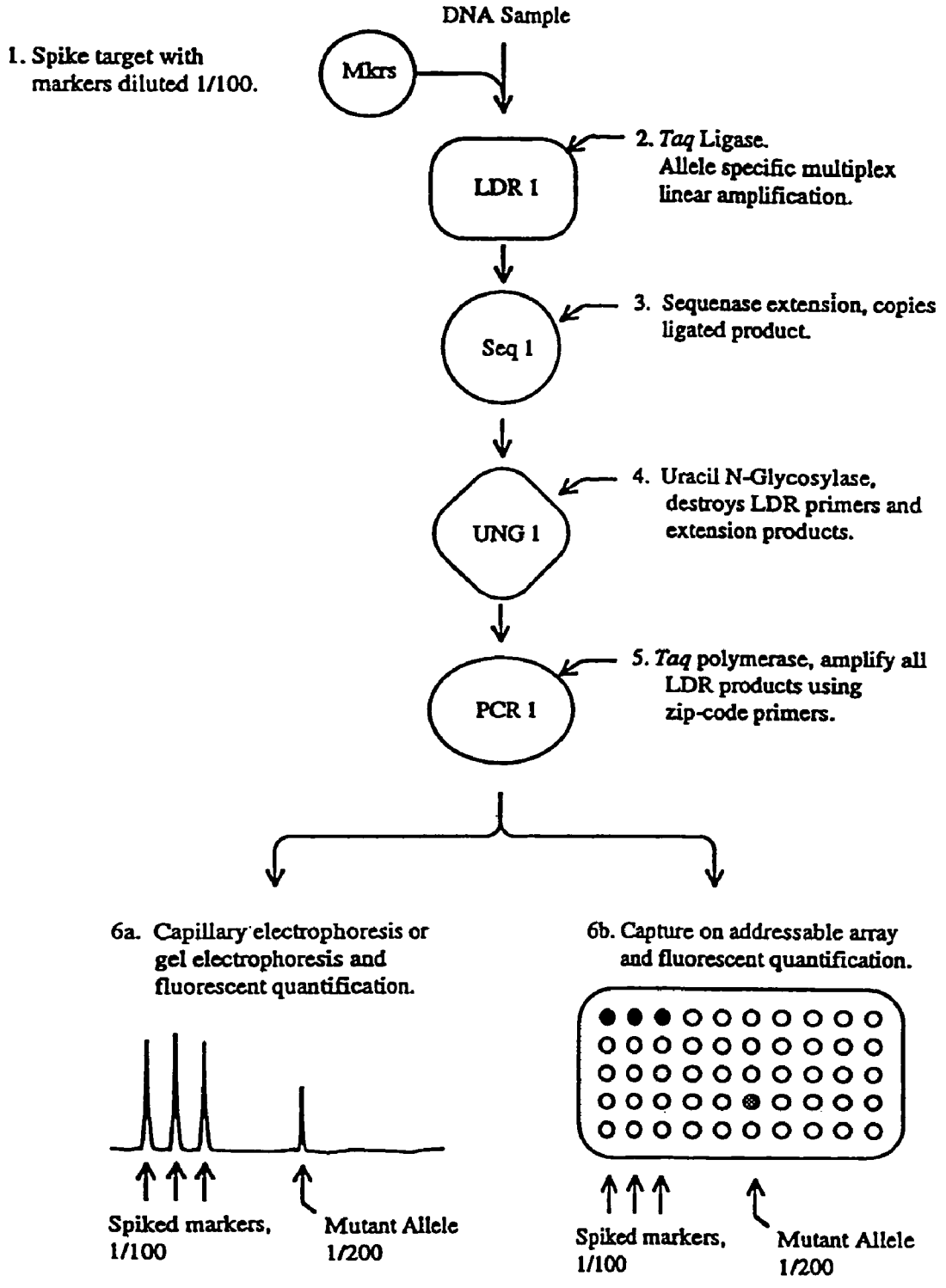

DNA Sample
1. Spike target with markers diluted 1/100.
Mkrs
LDR 1
2. Taq Ligase. Allele specific multiplex linear amplification.
Seq 1
3. Sequenase extension, copies ligated product.
UNG 1
4. Uracil N-Glycosylase, destroys LDR primers and extension products.
PCR 1
5. Taq polymerase, amplify all LDR products using zip-code primers.
6a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200
6b. Capture on addressable array and fluorescent quantification.
Spiked markers, 1/100
Mutant Allele 1/200

FIG. 19

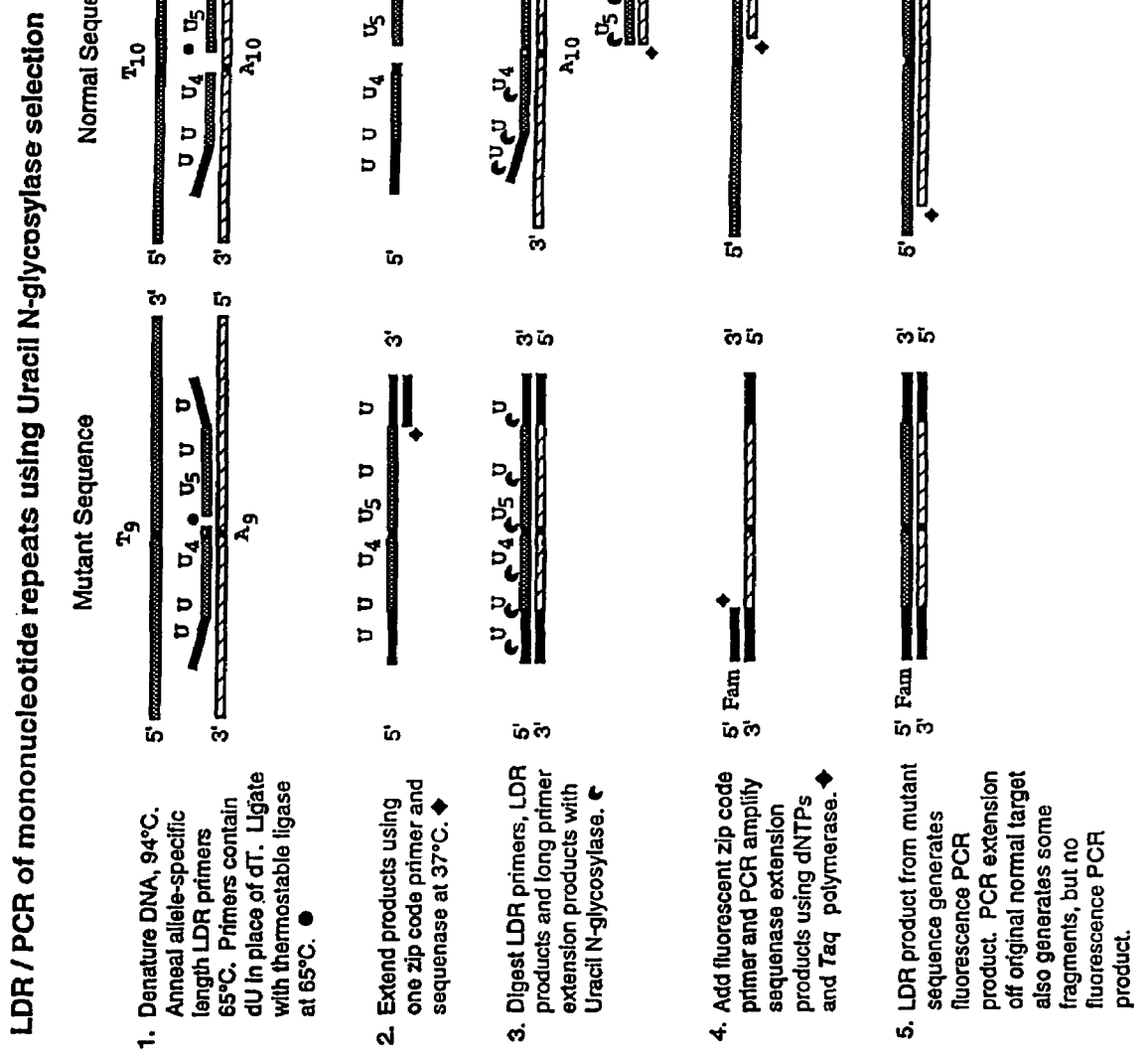

LDR / PCR of mononucleotide repeats using Uracil N-glycosylase selection
Mutant Sequence
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific length LDR primers 65°C. Primers contain dU in place of dT. Ligate with thermostable ligase at 65°C. ●
2. Extend products using one zip code primer and sequenase at 37°C. ◆
3. Digest LDR primers, LDR products and long primer extension products with Uracil N-glycosylase. ☛
4. Add fluorescent zip code primer and PCR amplify sequenase extension products using dNTPs and Taq polymerase. ✦
5. LDR product from mutant sequence generates fluorescence PCR product. PCR extension off original normal target also generates some fragments, but no fluorescence PCR product.
Fam

FIG. 20

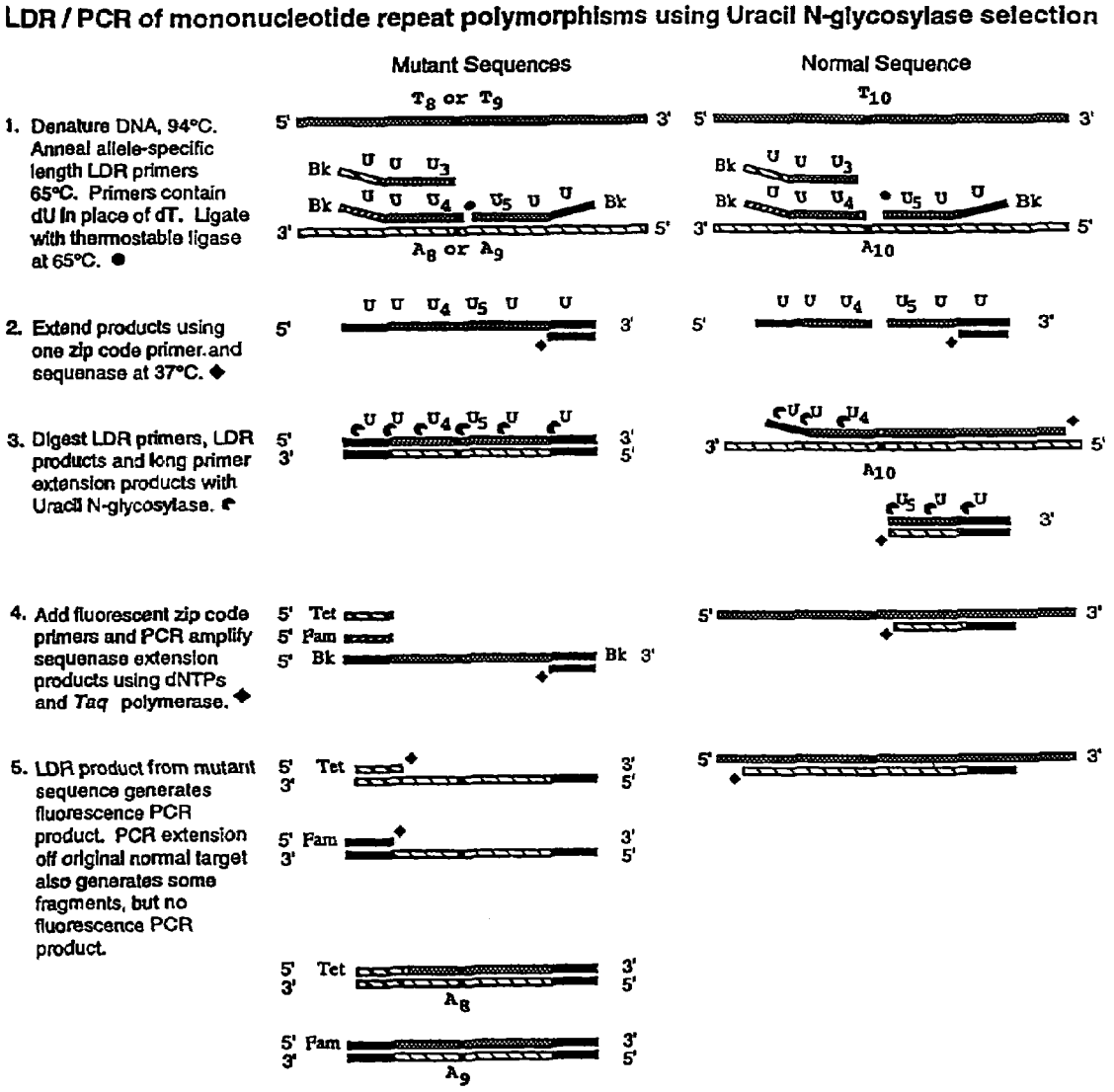

LDR / PCR of mononucleotide repeat polymorphisms using Uracil N-glycosylase selection
Mutant Sequences
Normal Sequence
1. Denature DNA, 94°C. Anneal allele-specific length LDR primers 65°C. Primers contain dU in place of dT. Ligate with thermostable ligase at 65°C. ●
2. Extend products using one zip code primer and sequenase at 37°C. ◆
3. Digest LDR primers, LDR products and long primer extension products with Uracil N-glycosylase.
4. Add fluorescent zip code primers and PCR amplify sequenase extension products using dNTPs and Taq polymerase. ◆
5. LDR product from mutant sequence generates fluorescence PCR product. PCR extension off original normal target also generates some fragments, but no fluorescence PCR product.
Tet
Fam
Bk

FIG. 21

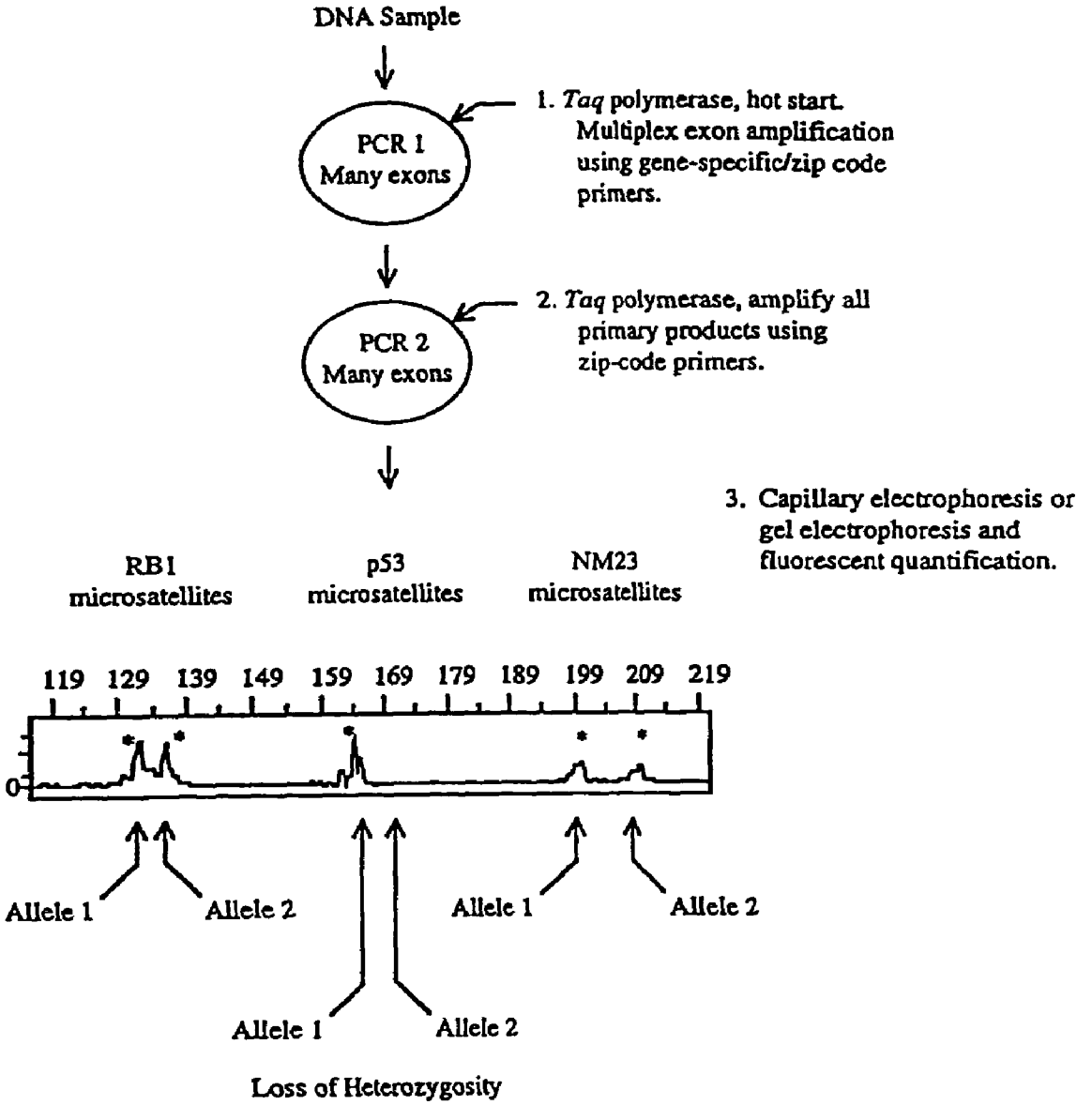

DNA Sample
PCR 1
Many exons
1. Taq polymerase, hot start. Multiplex exon amplification using gene-specific/zip code primers.
PCR 2
Many exons
2. Taq polymerase, amplify all primary products using zip-code primers.
3. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
RB1 microsatellites
p53 microsatellites
NM23 microsatellites
119 129 139 149 159 169 179 189 199 209 219
Allele 1
Allele 2
Allele 1
Allele 2
Allele 1
Allele 2
Loss of Heterozygosity

FIG. 22

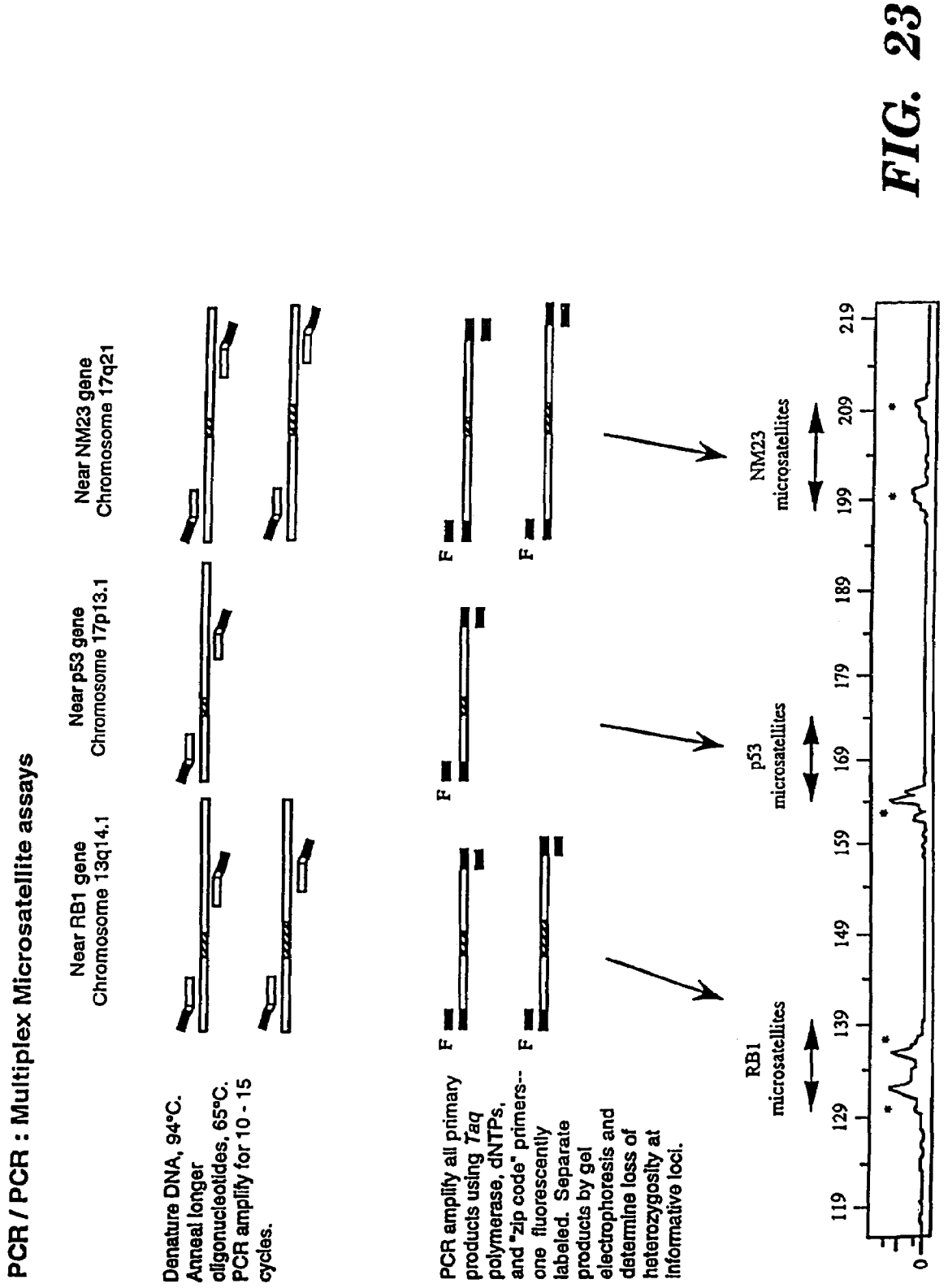

PCR / PCR : Multiplex Microsatellite assays
Near RB1 gene Chromosome 13q14.1
Near p53 gene Chromosome 17p13.1
Near NM23 gene Chromosome 17q21
1. Denature DNA, 94°C. Anneal longer oligonucleotides, 65°C. PCR amplify for 10 - 15 cycles.
2. PCR amplify all primary products using Taq polymerase, dNTPs, and "zip code" primers-- one fluorescently labeled. Separate products by gel electrophoresis and determine loss of heterozygosity at informative loci.
F
RB1 microsatellites
p53 microsatellites
NM23 microsatellites
119
129
139
149
159
169
179
189
199
209
219
0

FIG. 23

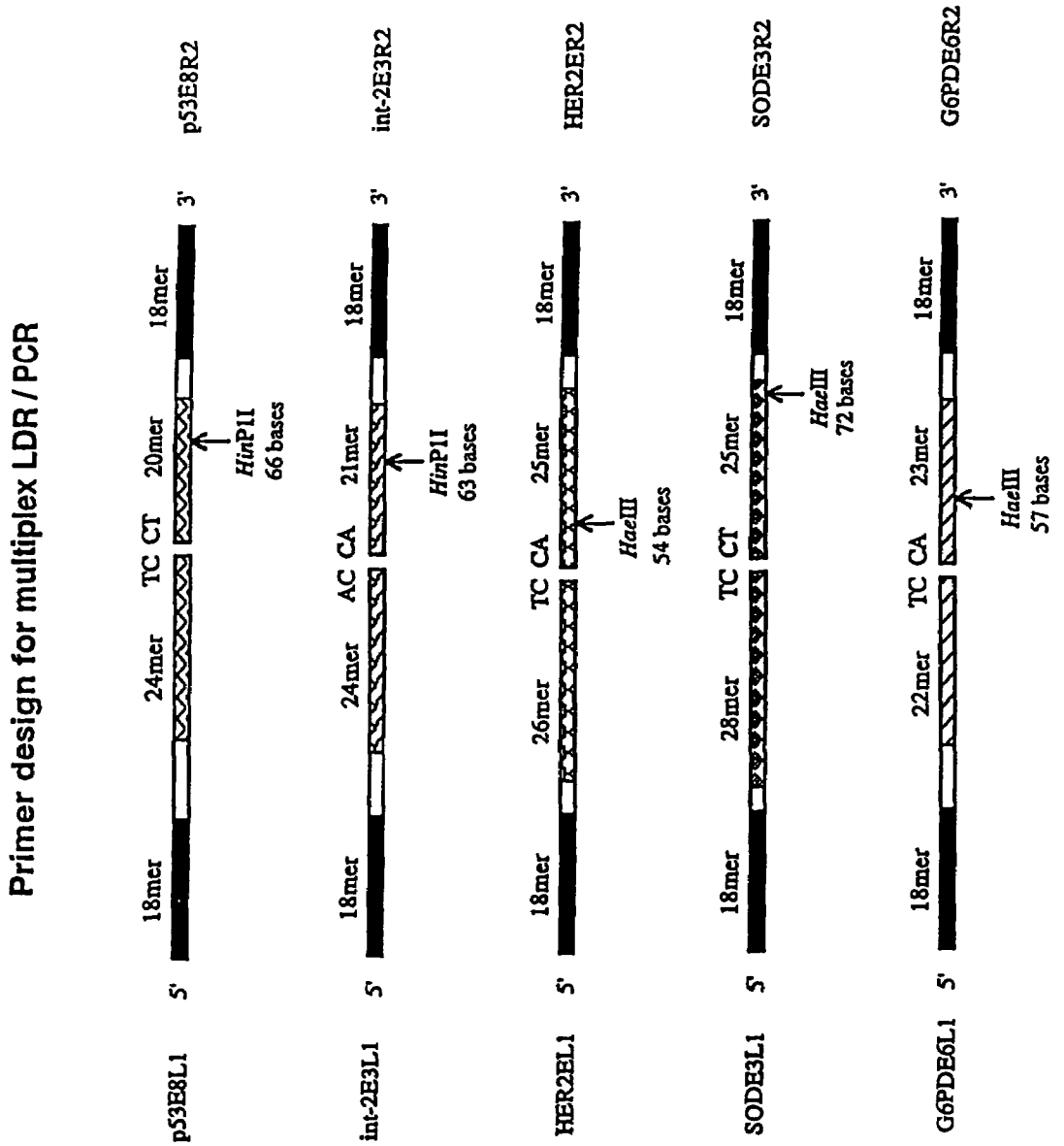

Primer design for multiplex LDR / PCR
p53E8L1
5'
18mer
24mer
TC CT
20mer
18mer
3'
p53E8R2
HinP1I
66 bases
int-2E3L1
5'
18mer
24mer
AC CA
21mer
18mer
3'
int-2E3R2
HinP1I
63 bases
HER2EL1
5'
18mer
26mer
TC CA
25mer
18mer
3'
HER2ER2
HaeIII
54 bases
SODE3L1
5'
18mer
28mer
TC CT
25mer
18mer
3'
SODE3R2
HaeIII
72 bases
G6PDE6L1
5'
18mer
22mer
TC CA
23mer
18mer
3'
G6PDE6R2
HaeIII
57 bases

FIG. 24

FIG. 25A

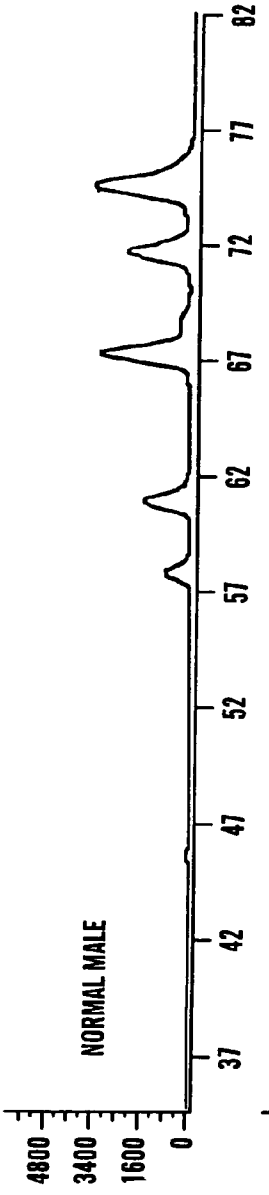


FIG. 25B

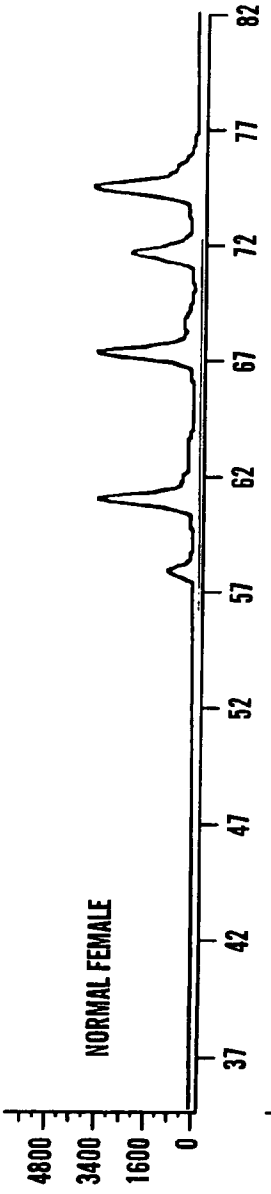


FIG. 25C

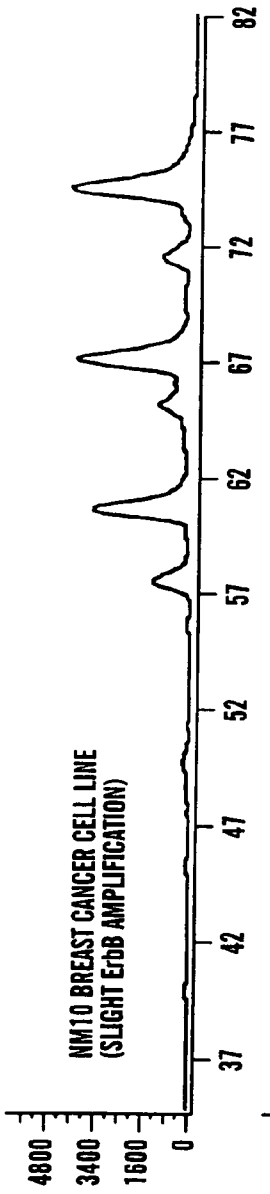


FIG. 25D

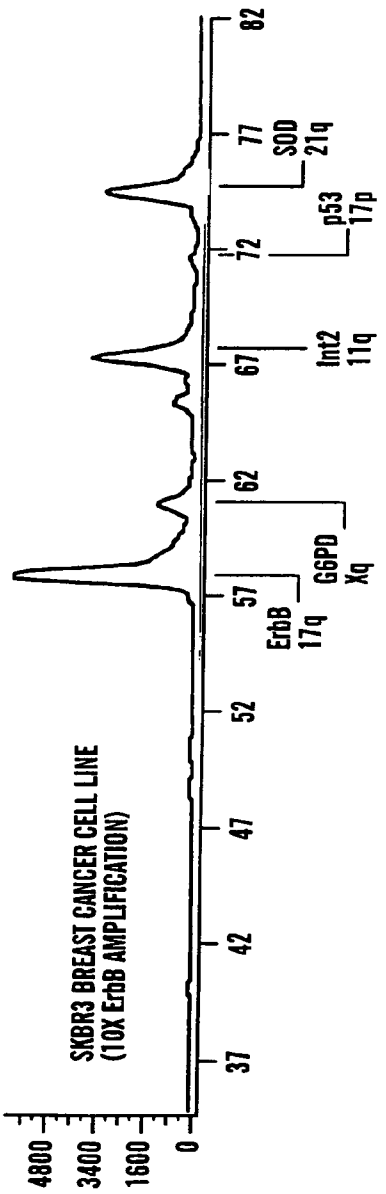


FIG. 26A

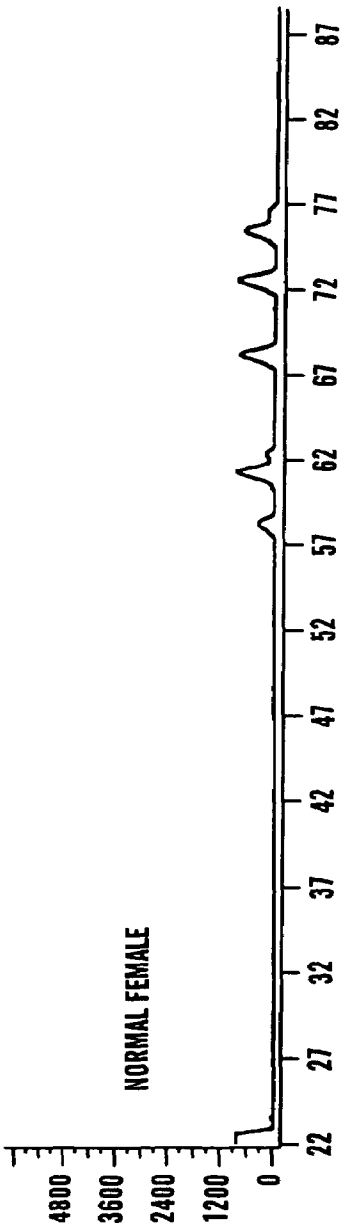


FIG. 26B

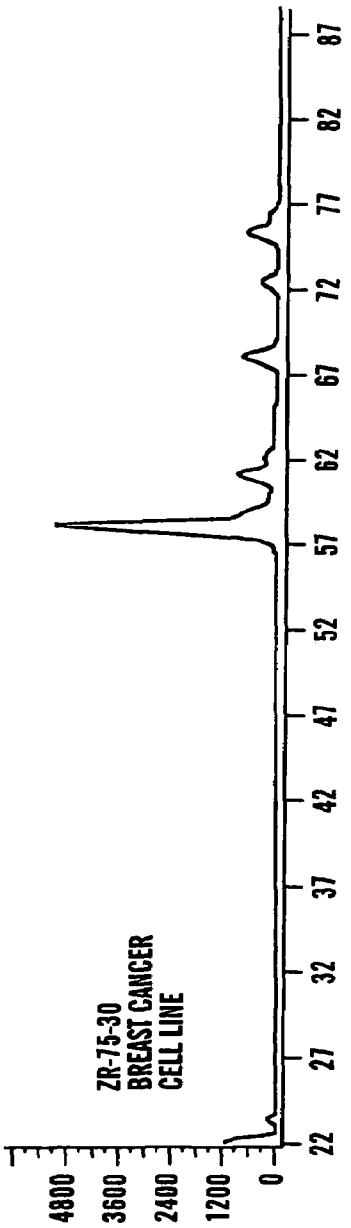


FIG. 26C

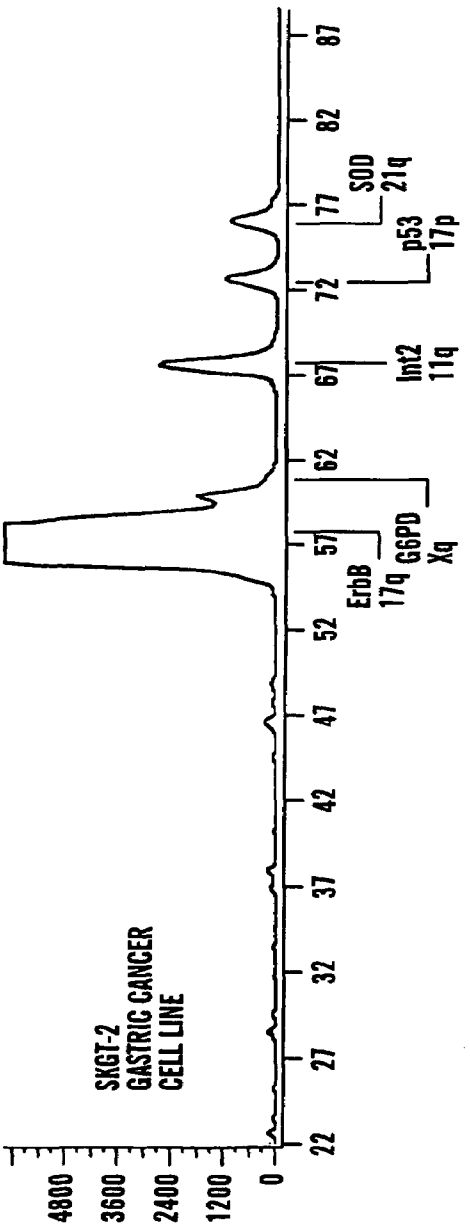


FIG. 27A

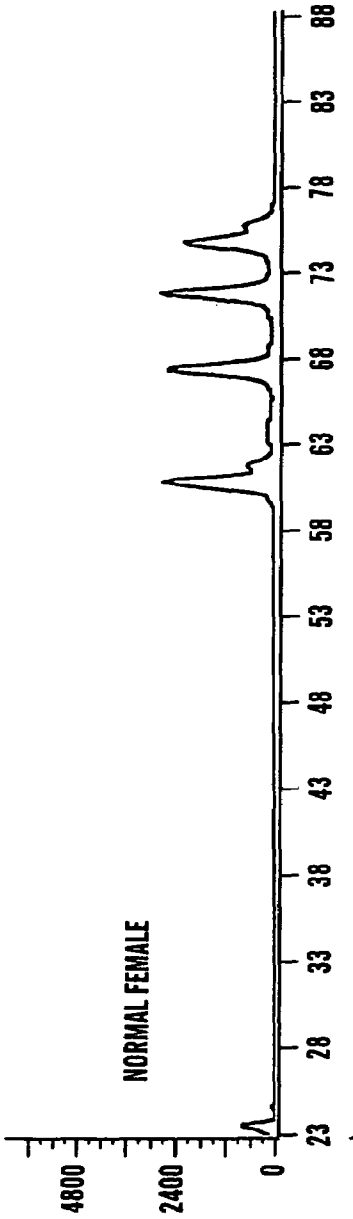


FIG. 27B

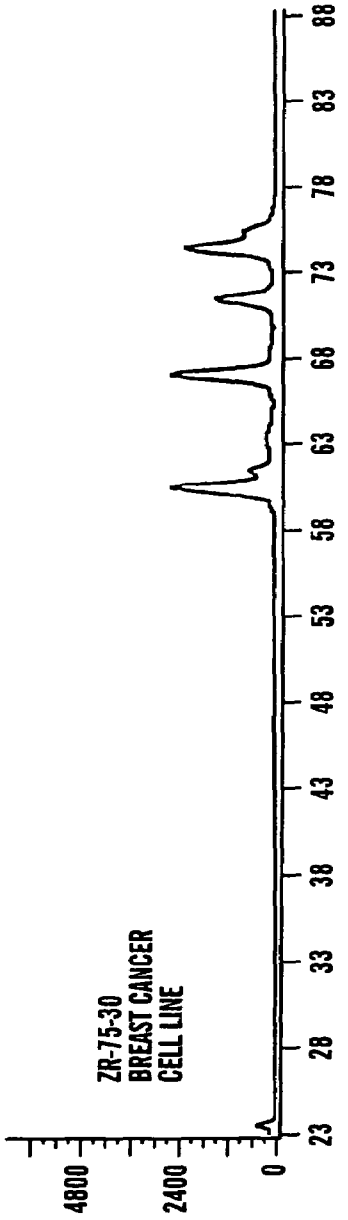
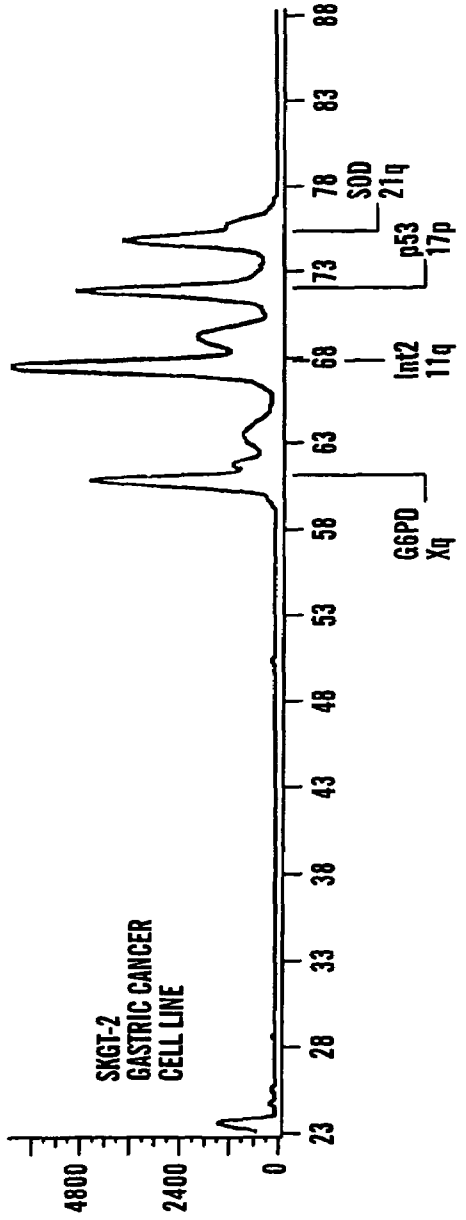


FIG. 27C



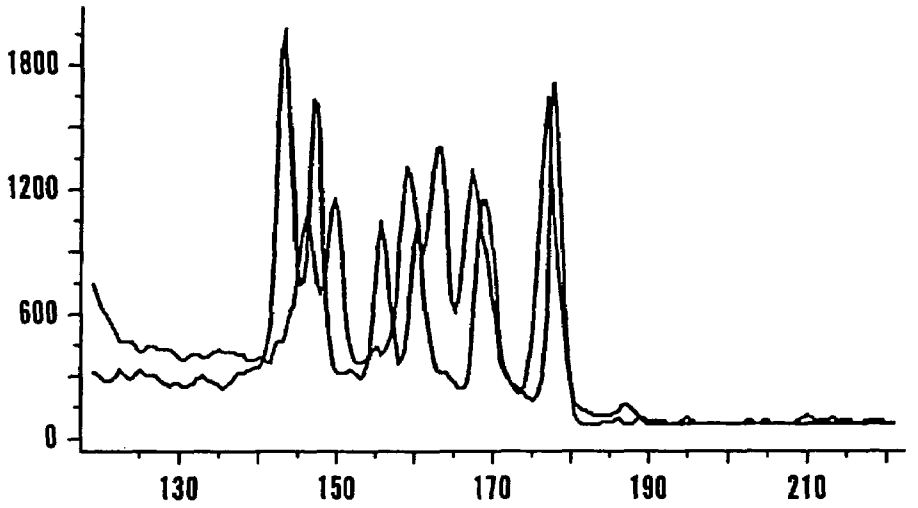


FIG. 28A

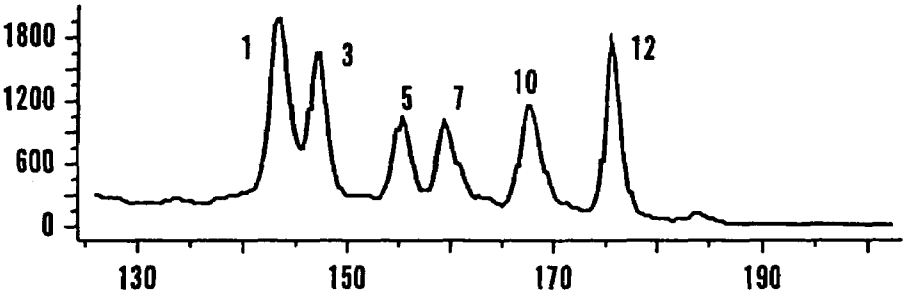


FIG. 28B

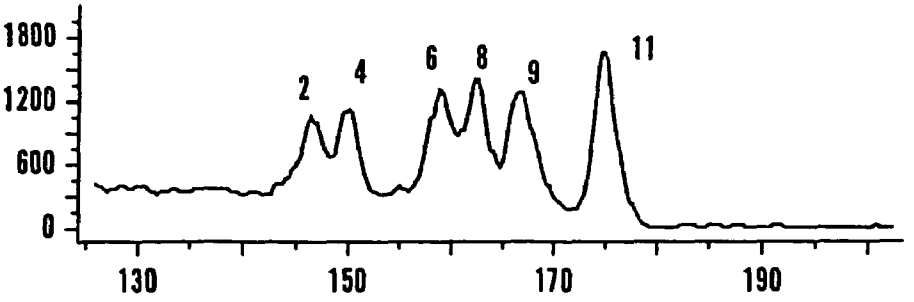


FIG. 28C

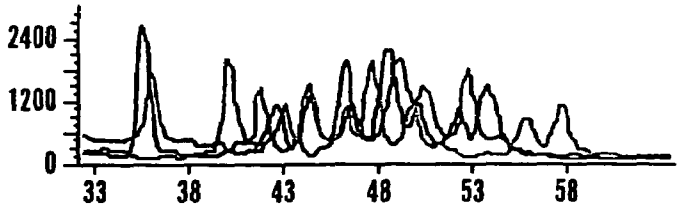


FIG. 29A

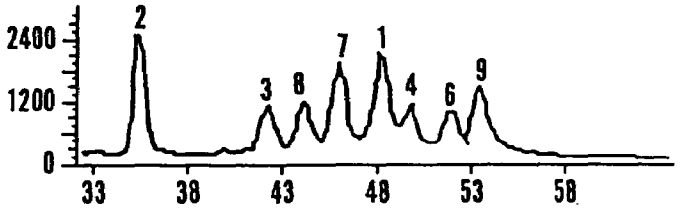


FIG. 29B

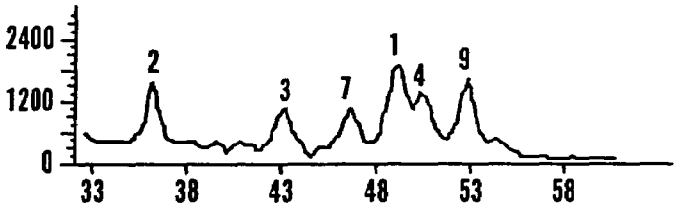


FIG. 29C

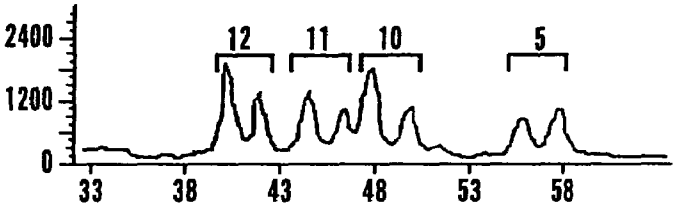


FIG. 29D

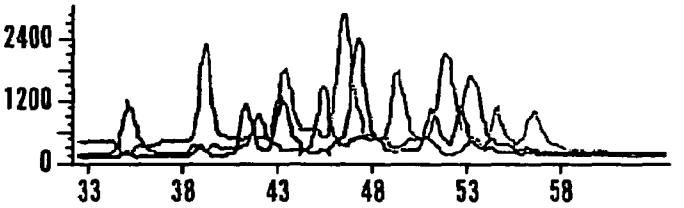


FIG. 29E

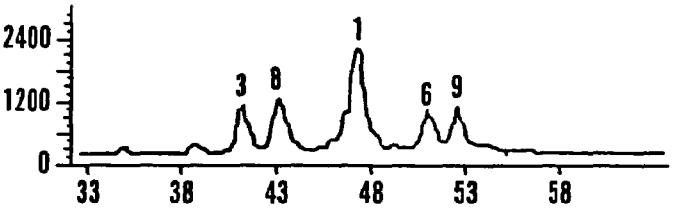


FIG. 29F

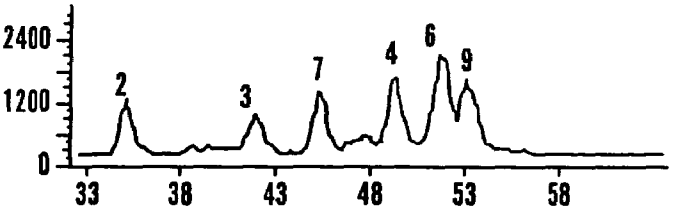


FIG. 29G

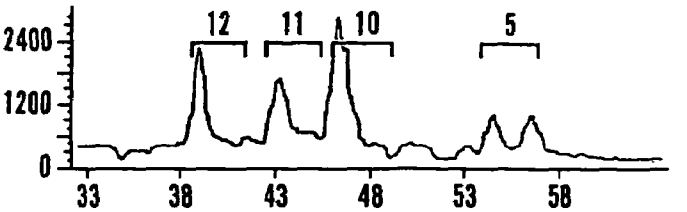


FIG. 29H

DETECTION OF NUCLEIC ACID SEQUENCE DIFFERENCES USING COUPLED LIGASE DETECTION AND POLYMERASE CHAIN REACTIONS

This application is a continuation of U.S. patent application Ser. No. 11/229,366, filed Sep. 16, 2005, which is a continuation of U.S. patent application Ser. No. 10/843,720, filed May 12, 2004, now U.S. Pat. No. 7,166,434, which is a continuation of U.S. patent application Ser. No. 09/918,156, filed Jul. 30, 2001, now U.S. Pat. No. 6,797,470 issued Sep. 28, 2004, which is a continuation of U.S. patent application Ser. No. 09/440,523, filed Nov. 15, 1999, now U.S. Pat. No. 6,268,148, issued Jul. 31, 2001, which is a divisional of U.S. patent application Ser. No. 08/864,473, filed May 28, 1997, now U.S. Pat. No. 6,027,889 issued Feb. 22, 2000, and claims the benefit of U.S. Provisional Patent Application Ser. No. 60/018,532, filed May 29, 1996, which are all hereby incorporated by reference in their entirety.

This invention was developed with government funding under National Institutes of Health Grant No. GM41337-06. The U.S. Government may retain certain rights.

FIELD OF THE INVENTION

The present invention relates to the detection of nucleic acid sequence differences using coupled ligase detection reaction ("LDR") and polymerase chain reaction ("PCR"). One aspect of the present invention involves use of a ligase detection reaction coupled to a polymerase chain reaction. Another aspect of the present invention relates to the use of a primary polymerase chain reaction coupled to a secondary polymerase chain reaction coupled to a ligase detection reaction. A third aspect of the present invention involves a primary polymerase chain reaction coupled to a secondary polymerase chain reaction.

BACKGROUND OF THE INVENTION

Multiplex Detection

Large-scale multiplex analysis of highly polymorphic loci is needed for practical identification of individuals, e.g., for paternity testing and in forensic science (Reynolds et al., Anal. Chem., 63:2-15 (1991)), for organ-transplant donor-recipient matching (Buyse et al., *Tissue Antigens*, 41:1-14 (1993) and Gyllensten et al., *PCR Meth. Appl*, 1:91-98 (1991)), for genetic disease diagnosis, prognosis, and prenatal counseling (Chamberlain et al., *Nucleic Acids Res.*, 16:11141-11156 (1988) and L. C. Tsui, *Human Mutat.*, 1:197-203 (1992)), and the study of oncogenic mutations (Hollstein et al., *Science*, 253:49-53 (1991)). In addition, the cost-effectiveness of infectious disease diagnosis by nucleic acid analysis varies directly with the multiplex scale in panel testing. Many of these applications depend on the discrimination of single-base differences at a multiplicity of sometimes closely spaced loci.

A variety of DNA hybridization techniques are available for detecting the presence of one or more selected polynucleotide sequences in a sample containing a large number of sequence regions. In a simple method, which relies on fragment capture and labeling, a fragment containing a selected sequence is captured by hybridization to an immobilized probe. The captured fragment can be labeled by hybridization to a second probe which contains a detectable reporter moiety.

Another widely used method is Southern blotting. In this method, a mixture of DNA fragments in a sample is fractionated by gel electrophoresis, then fixed on a nitrocellulose filter. By reacting the filter with one or more labeled probes under hybridization conditions, the presence of bands containing the probe sequences can be identified. The method is especially useful for identifying fragments in a restriction-enzyme DNA digest which contains a given probe sequence and for analyzing restriction-fragment length polymorphisms ("RFLPs").

Another approach to detecting the presence of a given sequence or sequences in a polynucleotide sample involves selective amplification of the sequence(s) by polymerase chain reaction. U.S. Pat. No. 4,683,202 to Mullis, et al. and R. K. Saiki, et al., *Science* 230:1350 (1985). In this method, primers complementary to opposite end portions of the selected sequence(s) are used to promote, in conjunction with thermal cycling, successive rounds of primer-initiated replication. The amplified sequence(s) may be readily identified by a variety of techniques. This approach is particularly useful for detecting the presence of low-copy sequences in a polynucleotide-containing sample, e.g., for detecting pathogen sequences in a body-fluid sanple.

More recently, methods of identifying known target sequences by probe ligation methods have been reported. U.S. Pat. No. 4,883,750 to N. M. Whiteley, et al., D. Y. Wu, et al., *Genomics* 4:560 (1989), U. Landegren, et al., *Science* 241:1077 (1988), and E. Winn-Deen, et al., *Clin. Chem.* 37:1522 (1991). In one approach, known as oligonucleotide ligation assay ("OLA"), two probes or probe elements which span a target region of interest are hybridized to the target region. Where the probe elements basepair with adjacent target bases, the confronting ends of the probe elements can be joined by ligation, e.g., by treatment with ligase. The ligated probe element is then assayed, evidencing the presence of the target sequence.

In a modification of this approach, the ligated probe elements act as a template for a pair of complementary probe elements. With continued cycles of denaturation, hybridization, and ligation in the presence of pairs of probe elements, the target sequence is amplified linearly, allowing very small amounts of target sequence to be detected and/or amplified. This approach is referred to as ligase detection reaction. When two complementary pairs of probe elements are utilized, the process is referred to as the ligase chain reaction which achieves exponential amplification of target sequences. F. Barany, "Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase," *Proc. Nat'l Acad. Sci. USA*, 88:189-93 (1991) and F. Barany, "The Ligase Chain Reaction (LCR) in a PCR World," *PCR Methods and Applications*, 1:5-16 (1991).

Another scheme for multiplex detection of nucleic acid sequence differences is disclosed in U.S. Pat. No. 5,470,705 to Grossman-et. al. where sequence-specific probes, having a detectable label and a distinctive ratio of charge/translational frictional drag, can be hybridized to a target and ligated together. This technique was used in Grossman, et. al., "High-density Multiplex Detection of Nucleic Acid Sequences: Oligonucleotide Ligation Assay and Sequence-coded Separation," *Nucl. Acids Res.* 22(21):4527-34 (1994) for the large scale multiplex analysis of the cystic fibrosis transmembrane regulator gene.

Jou, et. al., "Deletion Detection in Dystrophia Gene by Multiplex Gap Ligase Chain Reaction and Immunochromatographic Strip Technology," *Human Mutation* 5:86-93 (1995) relates to the use of a so called "gap ligase chain reaction" process to amplify simultaneously selected regions of multiple exons with the amplified products being read on

an immunochromatographic strip having antibodies specific to the different haptens on the probes for each exon.

There is a growing need (e.g., in the field of genetic screening) for methods useful in detecting the presence or absence of each of a large number of sequences in a target polynucleotide. For example, as many as 400 different mutations have been associated with cystic fibrosis. In screening for genetic predisposition to this disease, it is optimal to test all of the possible different gene sequence mutations in the subject's genomic DNA, in order to make a positive identification of "cystic fibrosis". It would be ideal to test for the presence or absence of all of the possible mutation sites in a single assay. However, the prior-art methods described above are not readily adaptable for use in detecting multiple selected sequences in a convenient, automated single-assay format.

Solid-phase hybridization assays require multiple liquid-handling steps, and some incubation and wash temperatures must be carefully controlled to keep the stringency needed for single-nucleotide mismatch discrimination. Multiplexing of this approach has proven difficult as optimal hybridization conditions vary greatly among probe sequences.

Developing a multiplex PCR process that yields equivalent amounts of each PCR product can be difficult and laborious. This is due to variations in the annealing rates of the primers in the reaction as well as varying polymerase extension rates for each sequence at a given Mg2+ concentration. Typically, primer, Mg2+, and salt concentrations, along with annealing temperatures are adjusted in an effort to balance primer annealing rates and polymerase extension rates in the reaction. Unfortunately, as each new primer set is added to the reaction, the number of potential amplicons and primer dimers which could form increase exponentially. Thus, with each added primer set, it becomes increasingly more difficult and time consuming to work out conditions that yield relatively equal amounts of each of the correct products.

Allele-specific PCR products generally have the same size, and an assay result is scored by the presence or absence of the product band(s) in the gel lane associated with each reaction tube. Gibbs et al., *Nucleic Acids Res*., 17:2437-2448 (1989). This approach requires splitting the test sample among multiple reaction tubes with different primer combinations, multiplying assay cost. PCR has also discriminated alleles by attaching different fluorescent dyes to competing allelic primers in a single reaction tube (F. F. Chehab, et al., *Proc. Natl. Acad. Sci. USA*, 86:9178-9182 (1989)), but this route to multiplex analysis is limited in scale by the relatively few dyes which can be spectrally resolved in an economical manner with existing instrumentation and dye chemistry. The incorporation of bases modified with bulky side chains can be used to differentiate allelic PCR products by their electrophoretic mobility, but this method is limited by the successful incorporation of these modified bases by polymerase, and by the ability of electrophoresis to resolve relatively large PCR products which differ in size by only one of these groups. Livak et al., *Nucleic Acids Res*., 20:4831-4837 (1989). Each PCR product is used to look for only a single mutation, making multiplexing difficult.

Ligation of allele-specific probes generally has used solid-phase capture (U. Landegren et al., *Science*, 241:1077-1080 (1988); Nickerson et al., *Proc. Natl. Acad. Sci. USA*, 87:8923-8927 (1990)) or size-dependent separation (D. Y. Wu, et al., *Genomics*, 4:560-569 (1989) and F. Barany, *Proc. Natl. Acad. Sci*., 88:189-193 (1991)) to resolve the allelic signals, the latter method being limited in multiplex scale by the narrow size range of ligation probes. Further, in a multiplex format, the ligase detection reaction alone cannot make enough product to detect and quantify small amounts of target sequences. The gap ligase chain reaction process requires an additional step—polymerase extension. The use of probes with distinctive ratios of charge/translational frictional drag for a more complex multiplex will either require longer electrophoresis times or the use of an alternate form of detection.

The need thus remains for a rapid single assay format to detect the presence or absence of multiple selected sequences in a polynucleotide sample.

Microsatellite Analysis

Tandem repeat DNA sequences known as microsatellites represent a very common and highly polymorphic class of genetic elements within the human genome. These microsatellite markers containing small repeat sequences have been used for primary gene mapping and linkage analysis. Weber, J. L. et al., *Am. J. Hum. Genet*. 44: 388-396 (1989); Weissenbach, J. et al., *Nature* (*London*) 359: 794-800 (1992). PCR amplification of these repeats allows rapid assessment for loss of heterozygosity and can greatly simplify procedures for mapping tumor suppressor genes. Ruppert, J. M., et al., *Cancer Res*. 53: 5093-94 (1993); van der Riet, et al., *Cancer Res*. 54: 1156-58 (1994); Nawroz, H., et al., *Cancer Res*. 54: 1152-55 (1994); Cairns, P., et al., *Cancer Res*. 54: 1422-24 (1994). More recently, they have been used to identify specific mutations in certain inherited disorders including Huntington disease, fragile X syndrome, myotonic dystrophy, spinocerebellar ataxia type I, spinobulbar muscular atrophy, and hereditary dentatorubral-pallidoluysian atrophy. The Huntington's Disease Collaborative Research Group Cell 72: 971-83 (1993); Kremer, E. J., et al., *Science* 252: 1711-14 (1991); Imbert, G., et al., *Nat. Genet*. 4: 72-76 (1993); Orr, H. T., et al., *Nat. Genet*. 4: 221-226 (1993); Biancalana, V., et al., *Hum. Mol Genet*. 1: 255-258 (1992); Chung, M.-Y., et al., *Nat. Genet*. 5: 254-258 (1993); Koide, R., et al., *Nat. Genet*. 6: 9-13 (1994). These inherited disorders appear to arise from the expansion of trinucleotide repeat units within susceptible genes. A more widespread microsatellite instability, demonstrated by expansion or deletion of repeat elements in neoplastic tissues, was first reported in colorectal tumors. Peinado, M. A., et al. *Proc. Natl. Acad. Sci. USA* 89: 10065-69 (1992); Ionov, Y., *Nature (London)* 363: 558-61 (1993); Thibodeau, S. N., et al., *Science* 260: 816-819 (1993) and later in several other tumor types (Risinger, J. I., *Cancer Res*. 53: 5100-03 (1993); Han, H.-J., et al., *Cancer Res*. 53: 5087-89 (1993); Peltomäki, P., *Cancer Res*. 53: 5853-55 (1993); Gonzalez-Zulueta, M., et al., *Cancer Res*. 53: 5620-23 (1993); Merlo, A., et al., *Cancer Res*. 54: 2098-2101 (1994)). In hereditary nonpolyposis colorectal carcinoma patients, this genetic instability is apparently due to inherited and somatic mutations in mismatch repair genes. Leach, F., et al., *Cell* 75: 1215-1225 (1993); Fishel, R., et al., *Cell* 75: 1027-38 (1993); Papadopoulos, N., et al., *Science* 263: 1625-29 (1994); Bronner, C. E., et al., *Nature* (*London*) 368: 258-61 (1994).

PCR is commonly used for microsatellite analysis in identifying both the appearance of new polymorphisms and the loss of heterozygosity in cancer detection. L. Mao, et. al., "Microsatellite Alterations as Clonal Markers for the Detection of Human Cancer," *Proc. Nat'l Acad. Sci USA* 91(21): 9871-75 (1994); L. Mao, et. al., "Molecular Detection of Primary Bladder Cancer by Microsatellite Analysis," *Science* 271:659-62 (1996); D. Radford, et. al., "Allelotyping of Ductal Carcinoma in situ of the Breast: Detection of Loci on 8p, 13q, 16k, 17p and 17q," *Cancer Res*. 55(15): 3399-05

(1995). In using PCR for such purposes, each PCR reaction is run individually and separated on a sequencing gel.

Although these references demonstrate that PCR has application to diagnosis and prognosis of certain cancers, this type of analysis is deficient, because it does not permit a high throughput and requires size separation. In addition, there are problems with PCR slippage, causing researchers to shift to tri-, tetra-, and higher nucleotide repeat units, making cancer detection more difficult.

Microsatellite markers have also been used for colon cancer detection (L. Cawkwell, et. al., "Frequency of Allele Loss of DCC, p53, RB1, WT1, NF1, NM23, and APC/MCC in Colorectal Cancer Assayed by Fluorescent Multiplex Polymerase Chain Reaction," *Br. J. Cancer* 70(5): 813-18 (1994)) and for genome mapping (P. Reed, et. al., "Chromosome-specific Microsatellite Sets for Fluorescent-Based, Semi-Automated Genome Mapping," *Nat. Genet*. 7(3): 390-95 (1994)). However, the key to such multiplex processes is the ability to perform them in a single reaction tube. Conventional multiplex microsatellite marker approaches require careful attention to primer concentrations and amplification conditions. Although PCR products can be pooled in sets, this requires a prerun on agarose gels to insure that the mixture has about equal amounts of DNA in each band.

Human Identification

PCR has also been used for human identification, such as paternity testing, criminal investigations, and military personnel identification. A. Syvanen et. al., "Identification of Individuals by Analysis of Biallelic DNA Markers, Using PCR and Solid-Phase Mini-Sequencing" *Am. J. Hum. Genet*. 52(1): 46-59 (1993) describes a mini-sequencing approach to human identification. The technique requires PCR-amplification of individual markers with at most 4 PCR reactions being carried out at a time in a single PCR tube. Mini-sequencing is carried out to determine individual polymorphisms.

Coupled Processes

G. Deng, et. al., "An Improved Method of Competitive PCR for Quantitation of Gene Copy Number," *Nucl. Acids Res*. 21: 484849 (1993)-describes a competitive PCR process. Here, two PCR steps are utilized with different sets of primers being used for each gene and its equivalent standard.

T. Msuih, et. al., "Novel, Ligation-Dependent PCR Assay for Detection of Hepatitis C. Virus in Serum," *J. Clin Microbio*. 34: 501-07 (1996) and Y. Park, et. al., "Detection of HCV RNA Using Ligation-Dependent Polymerase Chain Reaction in Formalin-Fixed Paraffin-Embedded Liver Tissue" (submitted) describe the use of a LDR/PCR process in work with RNA.

SUMMARY OF THE INVENTION

The present invention is directed to the detection of nucleic acid sequence differences using coupled LDR and PCR processes. The present invention can be carried out in one of the following 3 embodiments: (1) LDR coupled to PCR; (2) primary PCR coupled to secondary PCR coupled to LDR; and (3) primary PCR coupled to secondary PCR. Each of these embodiments have particular applicability in detecting certain characteristics. However, each requires the use of coupled reactions for multiplex detection of nucleic acid sequence differences where oligonucleotides from an early phase of each process contain sequences which may be used by oligonucleotides from a later phase of the process.

I. Primary PCR/Secondary PCR/LDR Process

One aspect of the present invention relates to a method for identifying two or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. This method involves a first polymerase chain reaction phase, a second polymerase chain reaction phase, and a ligase detection reaction phase. This process involves analyzing a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences.

In the first polymerase chain reaction phase, one or more primary oligonucleotide primer groups are provided. Each group comprises one or more primary oligonucleotide primer sets with each set having a first nucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion, and a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion. The first oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion and the second oligonucleotide primers of each set in the same group contain the same 5' upstream primer-specific portion. The oligonucleotide primers in a particular set are suitable for hybridization on complementary strands of a corresponding target nucleotide sequence to permit formation of a polymerase chain reaction product. However, there is a mismatch which interferes with formation of such a polymerase chain reaction product when the primary oligonucleotide primers hybridize to any other nucleotide sequence in the sample. The polymerase chain reaction products in a particular set may be distinguished from other polymerase chain reaction products in the same group or groups. The primary oligonucleotide primers, the sample, and the polymerase are blended to form a primary polymerase chain reaction mixture.

The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as substantially described above. During hybridization, target-specific portions of the primary oligonucleotide primers hybridize to the target nucleotide sequences. The extension treatment causes hybridized primary oligonucleotide primers to be extended to form primary extension products complementary to the target nucleotide sequence to which the primary oligonucleotide primers are hybridized.

Although the upstream secondary primer-specific portions of a primary oligonucleotide primer set are not present on the target DNA, their sequences are copied by the second and subsequent cycles of the primary polymerase-chain reaction phase. As a result, the primary extension products produced after the second cycle have the secondary primer-specific portions on their 5' ends and the complement of primer-specific portion on their 3' ends.

Next, there is a second polymerase chain reaction phase. This phase involves providing one or a plurality of secondary oligonucleotide primer sets. Each set has a first secondary oligonucleotide primer containing the same sequence as the 5' upstream portion of the first primary oligonucleotide primer, and a second secondary oligonucleotide primer containing the same sequence as the 5' upstream portion of the second primary oligonucleotide primer from the same primary oligonucleotide primer set as the first primary oligonucleotide complementary to the first secondary primer. A set of secondary oligonucleotide primers may be used to amplify all of the primary extension products in a

given group. The secondary oligonucleotide primers are blended with the primary extension products and the polymerase to form a secondary polymerase chain reaction mixture.

The secondary polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles having a denaturation treatment, a hybridization treatment, and an extension treatment, as substantially set forth above. During the hybridization treatment, the secondary oligonucleotide primers hybridize to the complementary sequences present on the primary extension products but not to the original target sequence. The extension treatment causes the hybridized secondary oligonucleotide primers to be extended to form secondary extension products complementary to the primary extension-products.

The last phase of this aspect of the present invention involves a ligase detection reaction process. Here, a plurality of oligonucleotide probe sets are provided where each set has a first oligonucleotide probe, having a secondary extension product-specific portion and a detectable reporter label, and a second oligonucleotide probe, having a secondary extension product-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a complementary secondary extension product-specific portion. However, there is a mismatch which interferes with such ligation when the oligonucleotide probes are hybridized to any other nucleotide sequence present in the sample. The ligation product of oligonucleotide probes in a particular set may be distinguished from either probe or other ligation products. The plurality of oligonucleotide probe sets, the secondary extension products, and a ligase are blended to form a ligase detection reaction mixture.

The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles having a denaturation treatment and hybridization treatment substantially as described above. In the hybridization treatment, the oligonucleotide probe sets hybridize at adjacent positions in a base-specific manner to the respective secondary extension products if present. As a result, adjacent probes ligate to one another to form a ligation product sequence containing the detectable reporter label and the secondary extension product-specific portions connected together. The oligonucleotide probe sets may hybridize to nucleotide sequences other than their respective complementary secondary extension products but do not ligate together due to the presence of one or more mismatches and individually separate during the denaturation treatment. Following the ligase detection reaction cycles, the reporter labels of the ligation product sequences are detected which indicates the presence of one or more target nucleotide sequences in the sample.

The primary PCR/secondary PCR/LDR process of the present invention provides significant advantages over the use of PCR alone in the multiplex detection of single nucleotide and tandem repeat polymorphism-s.

As noted above, the use of PCR alone requires heavy optimization of operating conditions in order to conduct multiplex detection procedures. Moreover, the quantity of oligonucleotide primers must be increased to detect greater numbers of target nucleotide sequences. However, as this occurs, the probability of target independent reactions (e.g., the primer-dimer effect) increases. In addition, the mutations must be known, false positives may be generated by polymerase extension off of normal template, closely-clustered sites due to interference of overlapping primers cannot undergo multiplex detection, single base or small insertions and deletions in small repeat sequences cannot be detected, and quantification of mutant DNA in high background of normal DNA is difficult. As a result, the number of target nucleotide sequences detected in a single multiplex PCR process is limited.

Direct sequencing requires enrichment of mutant samples in order to correct sequences, requires multiple reactions for large genes containing many exons, requires electrophoretic separation of products, is time consuming, and cannot be used to detect mutant DNA in less than 5% of background of normal DNA. When mini-sequencing, the mutation must be known, closely-clustered sites due to interference of overlapping primers cannot undergo multiplex detection, single base or small insertions and deletions in small repeat sequences cannot be detected, and four separate reactions are required. For allele-specific oligonucleotide hybridization ("ASO"), the mutation must be known, hybridization and washing conditions must be known, cross-reactivity is difficult to prevent, closely-clustered sites due to interference of overlapping primers cannot undergo multiplex detection, and mutant DNA cannot be detected in less than 5% of background of normal DNA. Primer-mediated RFLP requires electrophoretic separation to distinguish mutant from normal DNA, is of limited applicability to sites that may be converted into a restriction site, requires additional analysis to determine the nature of the mutation, and is difficult to use where the mutant DNA is in a high background of normal DNA. Single strand conformational polymorphism analysis ("SSCP") requires electrophoretic separation to distinguish mutant conformer from normal conformer, misses 30% of possible mutations, requires additional analysis to determine the nature of the mutation, and cannot distinguish mutations from silent polymorphisms. With dideoxynucleotide finger printing ("ddF"), it is difficult to detect mutations in a high background of normal DNA, electrophoretic separation is required to distinguish mutant conformer from normal conformer, additional analysis must be used to determine the nature of the mutation, and mutations cannot be distinguished from silent polymorphisms. Denaturing gradient gel electrophoresis ("DGGE") must electrophoretically separate mutant conformer from normal conformer, misses 30% of possible mutations, requires additional analysis to determine the nature of the mutation, cannot distinguish mutations from silent polymorphisms, and imposes technical challenges to reproducing previously achieved results. RNase mismatch cleavage requires additional analysis to determine the nature of the mutation, requires analysis of both strands to exclude RNase-resistant mismatches, and imposes difficulty in detecting mutations in a high background of normal DNA. Chemical mismatch cleavage cannot detect mutant DNA in less than 5% of background of normal DNA, and requires an analysis of both strands to detect all mutations. For T4 Endo VII mismatch cleavage, additional analysis is needed to determine the nature of the mutation, mutations cannot be distinguished from silent polymorphisms, endonuclease cleaves control DNA which necessitates careful interpretation of results, and it is difficult to detect mutations in a high background of normal DNA.

These problems are avoided in the primary PCR/secondary PCR/LDR process of the present invention which combines the sensitivity of PCR with the specificity of LDR. The primary PCR phase produces primary extension products with a secondary primer-specific portion. This initial phase is carried out under conditions effective to maximize production of primary extension products without obtaining the adverse effects sometimes achieved in a PCR-only process. In particular, the primary PCR phase of the present invention

is carried out with 15 to 20 PCR cycles and utilizes less primer than would be utilized in a PCR-only process. The primary PCR phase of the present invention produces extension products in a varied and unpredictable way, because some target nucleotide sequences will be amplified well, while others will not. However, in the secondary PCR phase, all of the primary extension products are amplified approximately equally, because they all have the same secondary primer-specific portions. Target nucleotide sequences originally present in the sample will not be amplified by the secondary PCR phase, because such sequences do not contain a secondary primer-specific portion. As a result, the primary PCR/secondary PCR/LDR process of the present invention is able to achieve multiplex detection of hundreds of nucleotide sequence differences in a single tube without undue customization of operating conditions for each particular sample being analyzed. Since the selection of mutant sequences is mediated by LDR rather than PCR, the primary PCR/secondary PCR/LDR process is less susceptible to false-positive signal generation. In addition, the primary PCR/secondary PCR/LDR process allows detection of closely-clustered mutations, detection of single base or small insertions and deletions in small repeat sequences, quantitative detection of less than 1% mutations in high background of normal DNA, and detection of ligation product sequences using addressable arrays. The only significant requirements are that the mutations be known and that a multitude of oligonucleotides be synthesized.

The ability to detect single nucleotide and tandem repeat polymorphisms is particularly important for forensic DNA identification and diagnosis of genetic diseases.

II. LDR/PCR Process

A second aspect of the present invention relates to a method for identifying one or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. This method has a ligase detection reaction phase followed by a polymerase chain reaction phase. This method involves providing a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences.

In the ligase detection reaction phase, one or more oligonucleotide probe sets are provided. Each set has a first oligonucleotide probe, having a target-specific portion and a 5' upstream primer-specific portion, and a second oligonucleotide probe, having a target-specific portion and a 3' downstream primer-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target nucleotide sequence. However, there is a mismatch which interferes with such ligation when they are hybridized to any other nucleotide sequence present in the sample. The sample, the plurality of oligonucleotide probe sets, and a ligase are blended together to form a ligase detection reaction mixture.

The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles. These cycles include a denaturation treatment and a hybridization treatment. In the denaturation treatment, any hybridized oligonucleotides are separated from the target nucleotide sequences. The hybridization treatment causes the oligonucleotide probe sets to hybridize at adjacent positions in a base-specific manner to their respective target nucleotide sequences if present in the sample. Once hybridized, the oligonucleotide probe sets ligate to one another to form a ligation product sequence. This product contains the 5' upstream primer-specific portion, the target-specific portions connected together, and the 3' downstream primer-specific portion. The ligation product sequence for each set is distinguishable from other nucleic acids in the ligase detection reaction mixture. The oligonucleotide probe sets hybridized to nucleotide sequences in the sample other than their respective target nucleotide sequences but do not ligate together due to a presence of one or more mismatches and individually separate during the subsequent denaturation treatment.

In the polymerase chain reaction, one or a plurality of oligonucleotide primer sets are provided. Each set has an upstream primer containing the same sequence as the 5' upstream primer-specific portion of the ligation product sequence and a downstream primer complementary to the 3' downstream primer-specific portion of the ligation product sequence, where one primer has a detectable reporter label. The ligase detection reaction mixture is blended with the one or a plurality of oligonucleotide primer sets and the polymerase to form a polymerase chain reaction mixture.

The polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles which include a denaturation treatment, a hybridization treatment, and an extension treatment. During the denaturation treatment, hybridized nucleic acid sequences are separated. The hybridization treatment causes primers to hybridize to their complementary primer-specific portions of the ligation product sequence. During the extension treatment, hybridized primers are extended to form extension products complementary to the sequences to which the primers are hybridized. In a first cycle of the polymerase chain reaction phase, the downstream primer hybridizes to the 3' downstream primer-specific portion of the ligation product sequence and is extended to form an extension product complementary to the ligation product sequence. In subsequent cycles the upstream primer hybridizes to the 5' upstream primer-specific portion of the extension product complementary to the ligation product sequence and the downstream primer hybridizes to the 3' downstream portion of the ligation product sequence.

Following the polymerase chain reaction phase of this process, the reporter labels are detected and the extension products are distinguished to indicate the presence of one or more target nucleotide sequences in the sample.

One embodiment of the LDR/PCR process of the present invention achieves improved results over the use of LDR alone in measuring the number of gene copies in a cell (i.e. gene dosage). When LDR alone is utilized, it is difficult to produce sufficient target copies which are needed ultimately to quantify a plurality of genes.

In another embodiment of the LDR/PCR process of the present invention, the LDR phase ligation product sequences are produced in a ratio proportional to the ratio of the genes from which they were derived within the sample. By incorporation of the same primer-specific portions in the oligonucleotide probes for the LDR phase, the PCR phase amplifies ligation product sequences to the same degree so that their proportionality is maintained. Target sequences originally found in the sample being analyzed are not amplified by the PCR phase, because such target sequences do not contain PCR primer-specific portions. In addition, since only the oligonucleotide primers for the PCR phase have reporter labels, only extension products with those labels will be detected.

Determination of variation in gene dosage is important in a number of biomedical applications.

Males differ in gene dosage from females for those genes located on the X chromosome, women having two copies while men have one copy. Women may be carriers of deletions along the X chromosome. If the deleted region of the X chromosome included one or more genes, then the woman has only one copy of these genes in the corresponding, non-deleted area of the other X chromosome. The result (having one copy of an X-linked gene) is similar to the situation in male cells and is usually tolerated without manifestations of an inherited disorder. However, if the woman's son inherits her deleted X chromosome, he will have no copies of the genes in the deletion region and suffer from the X-linked disorder related to the absence of the gene. The detection of chromosomal deletions, therefore, is one application of the LDR/PCR process of the present invention.

Congenital chromosomal disorders occur when a fertilized egg has an abnormal compliment of chromosomes. The most common congenital chromosomal disorder is Down Syndrome, which occurs when there is an additional chromosome 21 in each cell, designated 47; XX+21 or 47, XY+21. The LDR/PCR process of the present invention can be designed to identify congenital chromosomal disorders.

The LDR/PCR process of the present invention is also useful for distinguishing polymorphisms in mono-nucleotide and di-nucleotide repeat sequences. It will also be useful in distinguishing minor populations of cells containing unique polymorphisms for detection of clonality.

The LDR/PCR process can be used with both gel and non-gel (i.e. DNA array) technologies. It allows multiplex analysis of many gene amplifications and deletions simultaneously, allows quantitative analysis, and does not require an external standard. The only relatively minor challenge presented by the LDR/PCR process is that it is difficult to use in determining the boundaries of large deletions in chromosomes.

By contrast, microsatellite marker analysis cannot be used to detect small regions that are deleted or amplified, is not compatible with simultaneous detection of amplified regions, and depends on the availability of informative markers. Competitive PCR (i.e. differential PCR) cannot be used in a multiplex format to detect several deletions and amplifications simultaneously, and is not particularly accurate. Southern hybridization is time consuming, labor intensive, is not amenable to multiplexing due to the need for multiple steps for each probe tested, and requires large quantities of DNA. Fluorescent in situ hybridization ("FISH") requires specialized expertise, is time consuming, and requires large probes to analyze for suspected deleted or amplified regions. Thus, the LDR/PCR process of the present invention constitutes a significant advance over prior processes.

III. Primary PCR/Secondary PCR Process

A third aspect of the present invention also involves a method for identifying two or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in one or more target nucleotide sequences. This method involves subjecting a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences to two successive polymerase chain reaction phases.

For the first polymerase chain reaction phase, one or more primary oligonucleotide primer groups are provided where each group comprises two or more primary oligonucleotide primer sets. Each set has a first oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primary-specific portion, and a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion. The first oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion and the second oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion. The oligonucleotide primers in a particular set are suitable for hybridization on complementary strands of a corresponding target nucleotide sequence to permit formation of a polymerase chain reaction product. However, there is a mismatch which interferes with formation of such a polymerase chain reaction product when the primary oligonucleotide primers hybridize to any other nucleotide sequence present in the sample. The polymerase chain reaction products in a particular set may be distinguished from other polymerase chain reaction products with the same group or other groups. The primary oligonucleotide primers are blended with the sample and the polymerase to form a primary polymerase chain reaction mixture.

The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment as described above. During the hybridization treatment, the target-specific portion of a primary oligonucleotide primer is hybridized to the target nucleotide sequences. In the extension treatment, the hybridized primary oligonucleotide primers are extended to form primary extension products complementary to the target nucleotide sequence to which the primary oligonucleotide primer is hybridized.

Although the upstream secondary primer-specific portions of a primary oligonucleotide primer set are not present on the target DNA, their sequences are copied by the second and subsequent cycles of the primary polymerase chain reaction phase. As a result, the primary extension products produced after the second and subsequent cycles have the secondary primer-specific portions on their 5' ends and the complement of primer-specific portion on their 3' ends.

In the second polymerase chain reaction phase of this aspect of the present invention, one or a plurality of secondary oligonucleotide primer sets are provided. Each set has a first secondary primer having a detectable reporter label and containing the same sequence as the 5' upstream portion of a first primary oligonucleotide primer, and a second secondary primer containing the same sequence as the 5' upstream primer of the second primary oligonucleotide primer from the same primary oligonucleotide primer set as the first primary oligonucleotide complementary to the first secondary primer. A set of secondary oligonucleotide primers amplify the primary extension products in a given group. The secondary oligonucleotide primers are blended with the primary extension products and the polymerase to form a secondary polymerase chain reaction mixture.

The secondary polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as described above. In the hybridization treatment, the secondary oligonucleotide primers are hybridized to the primary extension products, while the extension treatment causes the hybridized secondary oligonucleotide primers to be extended to form secondary extension products complementary to the primary extension products. After subjecting the secondary polymerase chain reaction mixture to the two or more polymerase chain

reaction cycles, the labelled secondary extension products are detected. This indicates the presence of one or more target nucleotide sequences in the sample.

The primary PCR/secondary PCR process of the present invention provides significant advantages over Southern hybridization, competitive PCR, and microsatellite marker analysis in detecting nucleotide deletions which cause a loss of heterozygosity. Although Southern hybridization is more accurate than competitive PCR, it is quite labor intensive, requires large amounts of DNA, and neither technique can be multiplexed. Current multiplex microsatellite marker approaches require careful attention to primer concentrations and amplification conditions.

The primary PCR/secondary PCR process of the present invention overcomes these difficulties encountered in the prior art. In the primary PCR phase, the primary oligonucleotide primers flank dinucleotide or other repeat sequences and include a secondary primer-specific portion. The primary PCR phase is carried out at low concentrations of these primers to allow several loci to undergo amplification at the same time. The secondary PCR phase causes amplification to continue at the same rate with target-specific secondary primers being selected to space one set of microsatellite markers from the adjacent set. The primary PCR/secondary PCR process can be used to carry out multiplex detection in a single PCR tube and with single gel lane analysis.

This aspect of the present invention is useful in carrying out a microsatellite marker analysis to identify nucleotide deletions in a gene. Such multiplex detection can be carried out in a single reaction tube. However, the primary PCR/secondary PCR process cannot distinguish amplifications from deletions, so, when making such distinctions, this process must be used in conjunction with the above-described LDR/PCR process or a differential PCR process.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a flow diagram depicting a primary PCR/secondary PCR/LDR process for detection of germline mutations, such as point mutations, by electrophoresis or capture on an addressable array. Note that the term "zipcode" which appears in FIG. **1** and other drawings refers to a sequence specific to a subsequently used primer or probe but not to either the target sequence or other genome sequences.

FIG. **2** is a flow diagram depicting a primary PCR/secondary PCR/LDR process for detection of biallelic polymorphisms by electrophoresis or capture on an addressable array.

FIG. **3** is a flow diagram depicting a primary PCR/secondary PCR/LDR process for detection of cancer-associated mutations by electrophoresis or capture on an addressable array.

FIG. **4** is a schematic diagram depicting a primary PCR/secondary PCR/LDR process for detection of biallelic polymorphisms.

FIG. **5** is a schematic diagram depicting a primary PCR/secondary PCR/LDR process for detection of allelic differences using LDR oligonucleotide probes which distinguish all possible bases at a given site.

FIG. **6** is a schematic diagram depicting a primary PCR/secondary PCR/LDR process for detection of the presence of any possible base at two nearby sites using LDR oligonucleotide probes which distinguish all possible bases at a given site.

FIG. **7** is a schematic diagram depicting a primary PCR/secondary PCR/LDR process for detection of cancer-associated mutations at adjacent alleles.

FIG. **8** is a flow diagram depicting an LDR/PCR process with and without restriction endonuclease digestion using electrophoresis detection.

FIG. **9** is a flow diagram depicting an LDR/PCR process using detection on an addressable array using gene-specific addresses.

FIG. **10** is a schematic diagram depicting an LDR/PCR process for multiplex detection of gene amplifications and deletions.

FIG. **11** is a schematic diagram depicting an allele specific problem for an LDR/PCR process.

FIG. **12** is a schematic diagram depicting a solution for the allele specific problem for an LDR/PCR process which is shown in FIG. **11**.

FIG. **13** is a flow diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of biallelic polymorphisms by electrophoresis or capture on an addressable array.

FIG. **14** is a flow diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of cancer-associated mutations by electrophoresis or capture on an addressable array.

FIG. **15** is a schematic diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of allele specific mutations and polymorphisms.

FIG. **16** is a schematic diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of mononucleotide repeat polymorphisms.

FIG. **17** is a schematic diagram depicting an LDR/PCR process with an intermediate exonuclease digestion phase for detection of mononucleotide repeat polymorphisms which are in low abundance.

FIG. **18** is a flow diagram depicting the detection of polymorphisms using an LDR/PCR process with an intermediate sequenase extension phase and a uracil N-glycosylase digestion phase after the LDR phase and before the PCR phase and with detection by electrophoresis or an addressable array.

FIG. **19** is a flow diagram depicting the detection of cancer using an LDR/PCR process with an intermediate sequenase extension phase and a uracil N-glycosylase digestion phase after the LDR phase and before the PCR phase and with detection by electrophoresis or an addressable array.

FIG. **20** is a schematic diagram depicting detection of mononucleotide repeats using an LDR/PCR process with an intermediate sequenase amplification phase and a uracil N-glycosylase digestion phase after the LDR phase and before the PCR phase.

FIG. **21** is a schematic diagram depicting detection of mononucleotide repeat polymorphisms which are in low abundance using an LDR/PCR process with an intermediate sequenase amplification phase and a uracil N-glycosylase digestion phase after the LDR phase and before the PCR phase.

FIG. **22** is a flow diagram depicting a primary PCR/secondary PCR process for detection of microsatellite repeats.

FIG. **23** is a schematic diagram depicting a primary PCR/secondary PCR process for multiplex detection of insertions and deletions in microsatellite repeats.

FIG. **24** shows the design of LDR oligonucleotide probes for quantification of gene amplifications and deletions in an LDR/PCR process.

FIGS. **25**A-D show electropherogram results for an LDR/PCR process.

FIGS. **26**A-C show electropherogram results for an LDR/PCR process of ErbB, G6PD, Int2, p53, and SOD gene segments from normal human: female DNA and from DNA of the breast cancer cell line ZR-75-30 and the gastric carcinoma cell line SKGT-2. The ErbB gene is known to be amplified in the cancer cell lines. Target-specific ligation product sequences of 104 bp are generated in 10 cycles (94° C. for 30 sec, 65° C. for 4 min) of LDR using 500 femtomoles of each ligation primer, 50 ng of genomic DNA, 124 units of *Thermus thermophillus* ("Tth") ligase, 2 μl of 10× buffer (0.2 M Tris, pH 8.5 and 0.1 M $MgCl_2$), 2 μl of 10 mM NAD, and 1 μl of 200 mM DTT in a volume of 20 μl. The ligation products are proportionally amplified in 26 cycles (94° C. for 15 sec, 60° C. for 50 sec) of PCR by the addition of 30 μl of a solution containing 5 μl 10× Stoffel buffer (Perkin Elmer), 25 picomoles of each oligonucleotide primer, 2.5 units of Taq polymerase Stoffel fragment, and 8 μl of a solution 5 mM in each dNTP. After amplification, the products are digested with HaeIII and HinP1I to generate FAM-labeled products of 58 bp (ErbB (i.e. HER-2/neu/erbB oncogene)), 61 bp (G6PD), 67 bp (Int2 (i.e. int-2 oncogene)), 70 bp (p53) and 76 bp (SOD). These products are separated and analyzed on a 373A DNA sequencer using the Genescan 672 software package (Applied Biosystems, Inc., Foster City, Calif.). Results are displayed as electropherograms such that peak heights and areas reflect the amounts of the PCR products. In FIG. **26**A, the gene dosage determination for the five loci in normal human female DNA is shown. The peak heights and areas for G6PD, Int2, p53, and SOD are very similar. The peak height and area for ErbB is consistently small in normal genomic DNA. In FIG. **26**B, the peak height and area for ErbB are elevated when gene dosage is investigated in ZR-75-30, a cell line with known ErbB amplification. In FIG. **26**C, the gastric cell line, SKGT-2 shows dramatic amplification of the ErbB gene and a modest amplification of Int2. The G6PD gene peak may be embedded in the large ErbB peak.

FIGS. **27**A-C show electropherogram results for an LDR/PCR process to determine whether amplification of ErbB affected the relative peak heights of the other LDR oligonucleotide probes and PCR oligonucleotide primers for G6PD, Int2, p53, and SOD. In FIG. **27**A, the gene dosage determination for the four loci in normal human female DNA is shown. Peak heights and areas for G6PD, Int2, p53, and SOD are similar, as they were in the experiment using all five LDR primers. In FIG. **27**B, G6PD, Int2, and, SOD analyzed in the ZR-75-30 breast cancer cell line show similar relative peak heights, comparable to their appearance in normal female DNA. The peak height for p53 is reduced, suggesting the deletion of this gene in a portion of the cells in this cell line. In FIG. **27**C, in the gastric carcinoma cell line, SKGT-2, G6PD, and p53 show comparable peak heights. The Int2 peak height remains relatively high, as it was in the experiment using all five LDR oligonucleotide probes. Thus, the LDR and PCR amplification of each product appears to be independent of the other products during the course of the experiment.

FIGS. **28**A-C show electropherogram results for the PCR phase of a primary PCR/secondary PCR/LDR process. Multiplex PCR amplification of 12 loci using primary PCR oligonucleotide primers produces approximately equal amounts of product. Over 80 gene regions with single-base polymorphisms were identified from the Human Genome Database. Twelve of these (see Table 10 and FIGS. **29**A-H) were amplified in a primary PCR phase as follows: Long primary PCR oligonucleotide primers were designed to have gene-specific 3' ends and 5' ends complementary to one of two sets of secondary PCR oligonucleotide primers. The upstream primary PCR oligonucleotide primers were synthesized with either FAM (i.e. 6-carboxyfluorescein; fluorescent dye used in sequencing and mutation detection) or TET (i.e. tetrachlorinated-6-carboxytluorescein; fluorescent dye used in sequencing/mutation detection) fluorescent labels. All 24 base long primary PCR oligonucleotide primers were used at low concentration (2 picomole of each primer in 20 μl) in a 15 cycle primary PCR phase. After this, the two sets of secondary PCR oligonucleotide primers were added at higher concentrations (25 picomoles of each) and the secondary PCR phase was conducted for an additional 25 cycles. The products were separated on a 373 DNA Sequencer (Applied Biosystems). Panel A shows the electropherogram results for the FAM- and TET-labelled products combined. Panel B shows the FAM-labelled products alone. Panel C shows the TET-labelled products alone. The process produces similar amounts of multiplexed products without the need to adjust carefully primer concentrations or PCR conditions.

FIGS. **29**A-H show electropherogram results for the LDR phase of a primary PCR/secondary PCR/LDR process in detecting 12 biallelic genes for forensic identification. The primary and secondary PCR phases for the 12 polymorphic genes were performed as described in FIG. **28**A-C. However, the secondary PCR oligonucleotide primers were not fluorescently labelled. The secondary PCR process extension products were diluted into a ligase buffer containing 36 LDR oligonucleotide probes (one common and two discriminating primers for each locus). LDR oligonucleotide probe sets were designed in two ways: (i) allele-specific oligonucleotide probes were of the same length but contained either the FAM or TET label; or (ii) the allele-specific oligonucleotide probes were both labelled with HEX (i.e. hexachlorinated-6-carboxyfluorescein; fluorescent dye used in sequencing and mutation detection) but differed in length by two basepairs. After 20 cycles of the LDR phase, the ligation product sequences were resolved using a 10% polyacrylamide sequencing gel on a 373 DNA Sequencer (Applied Biosystems). Panel A and E show the 12 loci PCR/LDR profiles of two individuals. Panels B, C, and D show, respectively, the FAM, TET, and HEX data for the individual in panel A. Panels F, G, and H show, respectively, the FAM, TET, and HEX data for the individual in panel E. The individual in panel A is homozygous only at locus6 (ALDOB (i.e. aldolase B)) and locus 8 (IGF (i.e. insulin growth factor)). The individual in panel E is heterozygous only at loci 3 (C6 (i.e. complement component C6)), 5 (NF1 (i.e. neurofibromatosis)), 6 (ALDOB), and 8 (IGF). This demonstrates that the primary PCR/primary PCR/LDR process can simultaneously distinguish both homozygous and heterozygous genotypes at multiple positions.

DETAILED DESCRIPTION OF THE INVENTION

I. Primary PCR/Secondary PCR/LDR Process

One aspect of the present invention relates to a method for identifying two or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences.

This method involves a first polymerase chain reaction phase, a second polymerase chain reaction phase, and a ligase detection reaction phase. This process involves analyzing a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences.

In the first polymerase chain reaction phase, one or more primary oligonucleotide primer groups are provided. Each group comprises one or more primary oligonucleotide primer sets with each set having a first oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion, and a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion. The first oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion and the second oligonucleotide primers of each set in the same group contain the same 5' upstream primer-specific portion. The oligonucleotide primers in a particular set are suitable for hybridization on complementary strands of a corresponding target nucleotide sequence to permit formation of a polymerase chain reaction product. However, there is a mismatch which interferes with formation of such a polymerase chain reaction product when the primary oligonucleotide primers hybridize to any other nucleotide sequence present in the sample. The polymerase chain reaction products in a particular set may be distinguished from other polymerase chain reaction products in the same group or groups. The primary oligonucleotide primers, the sample, and the polymerase are blended to form a primary polymerase chain reaction mixture.

The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as substantially described above. During hybridization, target-specific portions of the primary oligonucleotide primers hybridize to the target nucleotide sequences. The extension treatment causes hybridized primary oligonucleotide primers to be extended to form primary extension products complementary, to the target nucleotide sequence to which the primary oligonucleotide primers are hybridized.

Although the upstream secondary primer-specific portions of a primary oligonucleotide primer set are not present on the target DNA, their sequences are copied by the second and subsequent cycles of the primary polymerase chain reaction phase. As a result, the primary extension products produced after the second cycle have the secondary primer-specific portions on their 5' ends and the complement of the primer-specific portion on their 3' ends.

Next, there is a second polymerase chain reaction phase. This phase involves providing one or a plurality of secondary oligonucleotide primer sets. Each set has a first secondary oligonucleotide primer containing the same sequence as the 5' upstream portion of the first primary oligonucleotide primer, and a second secondary oligonucleotide primer containing the same sequence as the 5' upstream portion of the second primary oligonucleotide primer from the same primary oligonucleotide primer set as the first primary oligonucleotide complementary to the first secondary primer. A set of secondary oligonucleotide primers may be used to amplify all of the primary extension products in a given group. The secondary oligonucleotide primers are blended with the primary extension products and the polymerase to form a secondary polymerase chain reaction mixture.

The secondary polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles having a denaturation treatment, a hybridization treatment, and an extension treatment, as substantially set forth above. During the hybridization treatment, the secondary oligonucleotide primers hybridize to complementary sequences present on the primary extension products but not to the original target sequence. The extension treatment causes the hybridized secondary oligonucleotide primers to be extended to form secondary extension products complementary to the primary extension products.

The last phase of this aspect of the present invention involves a ligase detection reaction process. Here, a plurality of oligonucleotide probe sets are provided where each set has a first oligonucleotide probe, having a secondary extension product-specific portion and a detectable reporter label, and a second oligonucleotide probe, having a secondary extension product-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a complementary secondary extension product-specific portion. However, there is a mismatch which interferes with such ligation when the oligonucleotide probes are hybridized to any other nucleotide sequence present in the sample. The ligation product of oligonucleotide probes in a particular set may be distinguished from either individual probes or other ligation products. The plurality of oligonucleotide probe sets, the secondary extension products, and a ligase are blended to form a ligase detection reaction mixture.

The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles having a denaturation treatment and hybridization treatment substantially as described above. In the hybridization treatment, the oligonucleotide probe sets hybridize at adjacent positions in a base-specific manner to the respective secondary extension products if present. As a result, adjacent probes ligate to one another to form a ligation product sequence containing the detectable-reporter label and the secondary extension product-specific portions connected together. The oligonucleotide probe sets may hybridize to nucleotide sequences other than the respective complementary secondary extension products but do not ligate together due a presence of one or more mismatches and individually separate during the denaturation treatment. Following the ligation detection reaction cycles, the reporter labels of the ligation product sequences are detected which indicates the presence of one or more target nucleotide sequences in the sample.

FIGS. **1**, **2**, and **3** show flow diagrams of the primary PCR/secondary PCR/LDR process of the present invention utilizing-either of two detection procedures. One alternative involves use of capillary electrophoresis or gel electrophoresis and a fluorescent quantification procedure. Alternatively, detection can be carried out by capture on an array of capture oligonucleotide addresses and fluorescent quantification. FIG. **1** relates to detection of germline mutations (e.g., a point mutation), while FIG. **2** detects biallelic polymorphisms, and FIG. **3** shows the detection of cancer-associated mutations.

FIG. **1** depicts the detection of a germline point mutation. In step 1, after DNA sample preparation, multiple exons are subjected to primary PCR amplification using. Taq (i.e. *Thermus aquaticus*) polymerase under hot start conditions with oligonucleotide primers having a target-specific portion and a secondary primer-specific portion. At the end of the primary PCR phase, Taq polymerase may be inactivated by heating at 100° C. for 10 min or by a freeze/thaw step. The products of the primary PCR amplification phase are then

subjected in step 2 to secondary PCR amplification using Taq polymerase under hot start conditions with the secondary oligonucleotide primers. At the end of the secondary PCR phase, Taq polymerase may be inactivated by heating at 100° C. for 10 min or by a freeze/thaw step. In step **3**, products of the secondary PCR phase are then diluted 20-fold into fresh LDR buffer containing LDR oligonucleotide probes containing allele-specific portions and common portions. step **4** involves the LDR phase of the process which is initiated by addition of Taq ligase under hot start conditions. During LDR, oligonucleotide probes ligate to their adjacent oligonucleotide probes only in the presence of target sequence which gives perfect complementarity at the junction site. The products may be detected in two different formats. In the first format 5a, fluorescently-labeled LDR probes contain different length poly A or hexaethylene oxide tails. Thus, each ligation product sequence (resulting from ligation of two probes hybridized on normal DNA) will have a slightly different length and mobility such that several ligation product sequences yield a ladder of peaks. Thus, each ligation product (resulting from ligation of two probes hybridized on normal DNA) will have a slightly different length and mobility such that several ligation product sequences yield a ladder of peaks. A germline mutation would generate a new peak on the electropherogram. Alternatively, the LDR probes may be designed such that the germline mutation ligation product sequence migrates with the same mobility as a normal DNA ligation product sequence, but it is distinguished by a different fluorescent reporter. The size of the new peak will approximate the amount of the mutation present in the original sample; 0% for homozygous normal, 50% for heterozygous carrier, or 100% for homozygous mutant. In the second format 5b, each allele-specific probe contains e.g., 24 additional nucleotide bases on their 5' ends. These sequences are unique addressable sequences which will specifically hybridize to their complementary address sequences on an addressable array. In the LDR reaction, each allele-specific probe can ligate to its adjacent fluorescently labeled common probe in the presence of the corresponding target sequence. Ligation product sequences corresponding to wild type and mutant alleles are captured on adjacent addresses on the array. Unreacted probes are washed away. The black dots indicate 100% signal for the wild type allele. The white dots indicate 0% signal for the mutant alleles. The shaded dots indicate the one position of germline mutation, 50% signal for each allele.

FIG. **2** depicts the detection of biallelic polymorphisms. In step **1**, after DNA sample preparation, multiple exons are subjected to primary PCR amplification using Taq polymerase under hot start-conditions with oligonucleotide primers having a target-specific portion and a secondary primer-specific portion. The products of the primary PCR amplification phase are then subjected in step **2** to secondary PCR amplification using Taq polymerase under hot start conditions with the secondary oligonucleotide primers. At the end of the secondary PCR phase, Taq polymerase may be inactivated by heating at 100° C. for 10 min or by a freeze/thaw step. In step **3**, products of the secondary PCR phase are then diluted 20-fold into fresh LDR buffer containing LDR oligonucleotide probes containing allele-specific portions and common portions. step **4** involves the LDR phase of the process which is initiated by addition of Taq ligase under hot start conditions. During LDR, oligonucleotide probes ligate to their adjacent oligonucleotide probes only in the presence of target sequence which gives perfect complementarity at the junction site. In the first format 5a, fluorescently-labeled LDR probes contain different length poly A or hexaethylene oxide tails. Each ligation product sequence will have a slightly different length and mobility, such that several LDR products yield a ladder of peaks. Alternatively, the LDR probes may be designed such that the ligation products for polymorphic alleles migrate at the same position but are distinguished by different fluorescent reporter groups. The size of the peaks will approximate the amount of each allele. In the second format 5b, each oligonucleotide probe contains unique addressable sequences with e.g., 24 additional nucleotide bases on their 5' ends. These sequences will specifically hybridize to their complementary address sequences on an array of capture oligonucleotides. In the LDR phase, each allele-specific probe can ligate to its adjacent fluorescently labeled common probe in the presence of corresponding target sequence. Ligation product sequences corresponding to each allele are captured on the array, while unligated oligonucleotide probes are washed away. The black dots indicate that both chromosomes have a given allele, the white dots show that neither chromosome has that allele, and the shaded dots indicate that one chromosome has a given allele.

FIG. **3** depicts the detection of cancer-associated mutation. In step **1**, after DNA sample preparation, multiple exons are subjected to primary PCR amplification using Taq polymerase under hot start conditions with oligonucleotide primers having a target-specific portion and a secondary primer-specific portion. The products of the primary PCR amplification phase are then subjected in step **2** to secondary PCR amplification using Taq polymerase under hot start conditions with the secondary oligonucleotide primers. At the end of the secondary PCR phase, Taq polymerase may be inactivated by heating at 100° C. for 10 min or by a freeze/thaw step. Fluorescent quantification of PCR products can be achieved using capillary or gel electrophoresis in step **3**. In step **4**, the products are spiked with a 1/100 dilution of marker DNA (for each of the fragments). This DNA is homologous to wild type DNA, except it contains a mutation which is not observed in cancer cells, but which may be readily detected with the appropriate LDR probes. In step **5**, the mixed DNA products in products of the secondary PCR phase are then diluted 20-fold into fresh LDR buffer containing LDR oligonucleotide probes containing allele-specific portions and common portions. step **6** involves the LDR phase of the process which is initiated by addition of Taq ligase under hot start conditions. During LDR, oligonucleotide probes ligate to their adjacent oligonucleotide probes only in the presence of target sequence which gives perfect complementarity at the junction site.

The products may be detected in the same two formats discussed above. In the format of step **7***a*, products are separated by capillary or gel electrophoresis, and fluorescent signals are quantified. Ratios of mutant peaks to marker peaks give the approximate amount of cancer mutations present in the original sample divided by 100. In the format of step **7***b*, products are detected by specific hybridization to complementary sequences on an addressable array. Ratios of fluorescent signals in mutant dots to marker dots give the approximate amount of cancer mutations present in the original sample divided by 100.

As shown in FIG. **4**, two DNA fragments of interest are treated with the primary PCR/secondary PCR/LDR process of the present invention. Initially, the double stranded DNA molecules are denatured to separate the strands. This is achieved by heating to a temperature of 80-105° C. Low concentrations of primary PCR oligonucleotide primers, containing a 3' target-specific portion (shaded area) and 5'

secondary primer-specific portion (black area), are then added and allowed to hybridize to the strands, typically at a temperature of 50-85° C. A thermostable polymerase (e.g., *Taq aquaticus* polymerase) is also added, and the temperature is then adjusted to 50-85° C. to extend the primer along the length of the nucleic acid to which the primer is hybridized. After the extension phase of the polymerase chain reaction, the resulting double stranded molecule is heated to a temperature of 80-105° C. to denature the molecule and to separate the strands. These hybridization, extension, and denaturation steps may be repeated a number of times to amplify the target to an appropriate level.

In the secondary PCR phase, the products of the primary PCR phase are blended with secondary PCR oligonucleotide primers and allowed to hybridize to one another, typically at a temperature of 50-85° C. The secondary oligonucleotide primers are usually used in higher concentrations than are the primary oligonucleotide primers. Taq polymerase is also added, and the temperature is then adjusted to 50-85° C. to extend the primer along the length of the primary PCR extension products to which the secondary oligonucleotide primer is hybridized. After the extension phase of the polymerase chain reaction, the resulting double stranded molecule is heated to a temperature of 80-105° C. to denature the molecule and to separate the strands. These hybridization, extension, and denaturation steps may be repeated a number of times to amplify the target to an appropriate level.

Once the secondary PCR phase of the process is completed, the ligation detection reaction phase begins, as shown in FIG. **4**. After denaturation of the target nucleic acid, if present as a double stranded DNA molecule, at a temperature of 80-105° C., preferably 94° C., ligation detection reaction oligonucleotide probes for one strand of the target nucleotide sequence are added along with a ligase (for example, as shown in FIG. **4**, a thermostable ligase like *Thermus aquaticus* ligase). The oligonucleotide probes are then allowed to hybridize to the target nucleic acid molecule and ligate together, typically, at a temperature of 45-85° C., preferably, 65° C. When there is perfect complementarity at the ligation junction, the oligonucleotides can be ligated together. Where the variable nucleotide is T or A, the presence of T in the target nucleotide sequence will cause the oligonucleotide probe with the F1 reporter label to ligate to the common oligonucleotide probe with the 5' poly A tail A_n, and the presence of A in the target nucleotide sequence will cause the oligonucleotide probe with the F2 reporter label to ligate to the common oligonucleotide probe with A_n. Similarly, where the variable nucleotide is A or G, the presence of T in the target nucleotide sequence will cause the oligonucleotide probe with F3AA reporter label (i.e. the F3 reporter label coupled to 2 additional bases forming a 5' poly A spacer) to ligate to the common oligonucleotide probe with the 5' poly A tail A_{n+4}, and the presence of C in the target nucleotide sequence will cause the oligonucleotide probe with the F3 reporter label to ligate to the common oligonucleotide probe with the 5' poly A tail A_{n+4}. Following ligation, the material is again subjected to denaturation to separate the hybridized strands. The hybridization/ligation and denaturation steps can be carried out through one or more cycles (e.g., 1 to 50 cycles) to amplify target signals. Equimolar ligation of both F3-labeled oligonucleotides indicates the individual is heterozygous for that locus, whereas ligation of only the F2 labeled oligonucleotides indicates the individual is homozygous for the other locus.

In FIG. **4**, the poly A_n and poly A_{n+4} tails are used where quantification is to be carried out by capillary or gel electrophoresis. The tails of differing length cause the corresponding different ligation product sequences to form bands at different locations in the gel or capillary. The presence of these bands at different locations permit the corresponding nucleotide differences in the DNA being analyzed to be identified. Although ligation product sequences can be distinguished based on the use of different reporter labels, the combination of different reporter labels and different length tails permits greater numbers of nucleotide differences to be distinguished. This is important for multiplex detection processes.

As noted above with respect to FIGS. **1** to **3**, detection can be carried out on an addressable array instead of with gel or capillary electrophoresis. The use of such addressable arrays require that the poly A tails on the LDR oligonucleotide probe not containing a reporter label (i.e. tails A_n and A_{n+4}) be replaced with different addressable array-specific oligonucleotide portions. As explained more fully infra, a solid support is provided with an array of capture oligonucleotides, some of which are complementary to the different addressable array-specific oligonucleotide portions. Hybridization of these portions to their complementary capture oligonucleotide probes indicates the presence of a corresponding nucleotide: difference.

FIG. **5** is a schematic diagram of a primary PCR/secondary PCR/LDR process, in accordance with the present invention, where any possible base in 2 DNA molecules of interest are distinguished. The primary and secondary PCR processes are carried out in substantially the same way as described for FIG. **4**. Appearance of fluorescent reporter labels F1, F2, F3, and F4 in conjunction with the left hand DNA molecule indicates the presence of the A, G, C, and T alleles in the DNA molecule, respectively. As shown in FIG. **5**, equal amounts of the F1 and F3 reporter labels indicates that the individual in question is heterozygous for the A and C alleles. With respect to analysis of the right hand DNA molecule in FIG. **5**, the same reporter label is used to indicate the presence of the different alleles; however, on each oligonucleotide probe with the distinguishing bases, there are different 5' poly A tails. More particularly, a 2 unit poly A tail, a 4 unit poly A tail, a 6 unit poly A tail, and an 8 unit poly A tail correspond to the T, C, G, and A alleles in the DNA molecule, respectively. As shown in FIG. **5**, equal amounts of the F1 reporter label with the A_6 and A_4 tails indicates that the individual in question is heterozygous for the G and C alleles.

FIG. **6** is a schematic diagram of a primary PCR/secondary PCR/LDR process, in accordance with the present invention, for detecting the presence of any possible base at two nearby sites in DNA molecules of interest. The primary and secondary PCR phases are carried out in substantially the same way as described for FIG. **4**. Here, the LDR probes are able to overlap, yet are still capable of ligating provided there is perfect complementarity at the junction. This distinguishes LDR from other approaches, such as allele-specific PCR where overlapping primers would interfere with one another. In FIG. **6**, the discriminating oligonucleotide probes contain the reporter label with the discriminating base on the 3' end of these probes. The poly A tails are on the 3' end of common oligonucleotide probes. In the left hand DNA molecule, the presence of equal amounts of ligation product sequences with reporter labels F1 and F3 shows that the individual in question is heterozygous for the A and C alleles in the first position. Similarly, in the second position for the left hand DNA molecule, the presence of ligation product sequences with reporter labels F2, F3, and F4 shows that the individual in question is heterozygous for

the G, C, and T alleles. Turning to the right hand DNA molecule, the presence of equal amounts of ligation product sequences with reporter label F1 having the A_6 and A_4 tails indicates that at the first position, the individual in question is heterozygous for the G and C alleles. In the second position for the right hand DNA molecule, the presence of the equal amounts of ligation product sequences with reporter label F1 having the A_8 and A_2 tails indicates that the individual in question is heterozygous for the A and T alleles.

FIG. **7** is a schematic diagram depicting the use of the primary PCR/secondary PCR/LDR process of the present invention to detect a low abundance mutation in the presence of an excess of normal sequence. Here, in the left hand DNA molecule is codon 12 of the K-ras gene, sequence GGT which codes for glycine ("Gly"). A small percentage of the cells contain the G to A mutation in GAT, which codes for aspartic acid ("Asp"). The LDR probes for wild-type (i.e. normal) sequences are missing from the reaction. If the normal LDR probes (with the discriminating base being G) were included, they would ligate to the common probes and overwhelm any signal coming from the mutant target. Instead, as shown in FIG. **7**, the existence of a ligation product sequence with fluorescent label F1 and the A_{n+2} tail indicates the presence of the aspartic acid encoding mutant. In the right hand DNA molecule, FIG. **7** shows codon 61 of the K-ras gene sequence CAG which codes for glutamine ("Gln"). A small percentage of the cells contain the C to G mutation in GAG, which codes for glutamic acid ("Glu"). Again, the LDR oligonucleotide probes do not include the C and A bases found in the wild type form to avoid overwhelming the mutant signal. For this DNA molecule, the existence of a ligation product sequence with fluorescent label F2 and the A_{n+4} tail indicates the presence of the glutamic acid encoding mutant.

II. LDR/PCR Process

A second aspect of the present invention relates to a method for identifying one or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in a plurality of target nucleotide sequences. This method has a ligase detection reaction phase followed by a polymerase chain reaction phase. This method involves providing a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences.

In the ligase detection reaction phase, one or more of oligonucleotide probe sets are provided. Each set has a first oligonucleotide probe, having a target-specific portion and a 5' upstream primer-specific portion, and a second oligonucleotide probe, having a target-specific portion and a 3' downstream primer-specific portion. The oligonucleotide probes in a particular set are suitable for ligation together when hybridized adjacent to one another on a corresponding target nucleotide sequence. However, there is a mismatch which interferes with such ligation when they are hybridized to any other nucleotide sequence present in the sample. The sample, the plurality of oligonucleotide probe sets, and a ligase are blended together to form a ligase detection reaction mixture.

The ligase detection reaction mixture is subjected to one or more ligase detection reaction cycles. These cycles include a denaturation treatment and a hybridization treatment. In the denaturation treatment, any hybridized oligonucleotides are separated from the target nucleotide sequences. The hybridization treatment causes the oligonucleotide probe sets to hybridize at adjacent positions in a base-specific manner to their respective target nucleotide sequences if present in the sample. Once hybridized, the oligonucleotide probe sets ligate to one another to form a ligation product sequence. This product contains the 5' upstream primer-specific portion, the target-specific portions connected together, and the 3' downstream primer-specific portion. The ligation product sequence for each set is distinguishable from other nucleic acids in the ligase detection reaction mixture. The oligonucleotide probe sets hybridized to nucleotide sequences in the sample other than their respective target nucleotide sequences but do not ligate together due to a presence of one or more mismatches and individually separate during the denaturation treatment.

In the polymerase chain reaction, one or a plurality of oligonucleotide primer sets are provided. Each set has an upstream primer containing the same sequence as the 5' upstream primer-specific portion of the ligation product sequence and a downstream primer complementary to the 3' downstream primer-specific portion of the ligation product sequence, where one primer has a detectable reporter label. The ligase detection reaction mixture is blended with the one or a plurality of oligonucleotide primer sets and the polymerase to form a polymerase chain reaction mixture.

The polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles which include a denaturation treatment, a hybridization treatment, and an extension treatment. During the denaturation treatment, hybridized nucleic acid sequences are separated. The hybridization treatment causes primers to hybridize to their complementary primer-specific portions of the ligation product sequence. During the extension treatment, hybridized primers are extended to form extension products complementary to the sequences to which the primers are hybridized. In a first cycle of the polymerase chain reaction phase, the downstream primer hybridizes to the 3' downstream primer-specific portion of the ligation product sequence and is extended to form an extension product complementary to the ligation product sequence. In subsequent cycles, the upstream primer hybridizes to the 5' upstream primer-specific portion of the extension product complementary to the ligation product sequence and the 3' downstream primer hybridizes to the 3' downstream portion of the ligation product sequence.

Following the polymerase chain reaction phase of this process, the reporter labels are detected and the extension products are distinguished to indicate the presence of one or more target nucleotide sequences in the sample.

FIG. **8** is a flow diagram depicting the LDR/PCR process of the present invention with or without restriction endonuclease digestion and using capillary electrophoresis detection. In step **1**, a DNA sample is mixed with Taq ligase and oligonucleotide probes containing a target-specific portion and a primer-specific portion. The mixture is subjected to an LDR process to produce ligation product sequences containing the ligated target-specific portions and the primer-specific portions. step **2** involves mixing the ligation product sequences with Taq polymerase and primers and subjecting the mixture to a PCR process. The next step is determined as a function of whether the ligation product sequences are the same or different sizes. Where the ligation product sequences are different sizes, step **3***a* is selected which involves subjecting the extension products from PCR to capillary electrophoresis or gel electrophoresis, either of which is followed by fluorescent quantification. Step **3***b* is utilized where the ligation product sequences are the same size and involves subjecting the extension products from the PCR phase to restriction endonuclease digestion. This gen-

erates digestion fragments of unique size which can be subjected to capillary electrophoresis or gel electrophoresis, followed by fluorescent quantification, according to step **4***b*. When step **3***a* is selected, the curve generated as a result of electrophoresis shows three ligation product sequences migrating at lengths of 104, 107, and 110, with the peak areas representing amplification of the Her-2 gene, loss of heterozygosity of the p53 gene, and the control SOD gene, respectively. The electrophoresis curve where steps **3***b* and **4***b* are used involves three ligation product sequence restriction fragments at lengths of 58, 70, and 76, with the peak areas representing amplification of the Her-2 gene, loss of heterozygosity of the p53 gene, and the control SOD gene, respectively.

As an alternative to FIG. **8**, FIG. **9** shows the LDR/PCR process of the present invention where, in step **3**, the extension products are captured on an array of capture oligonucleotide addresses. The capture oligonucleotide probes can be complementary to a nucleotide sequence across the ligation junction. The number of gene copies captured on the array of capture oligonucleotides is then determined by fluorescent quantification as compared with known controls. In FIG. **8**, such analysis of the array indicates ligation product sequences hybridizing to gene-specific addresses, where the fluorescent intensity represents amplification of the Her-2 gene, loss of heterozygosity of the p53 gene, and the control SOD gene, respectively.

FIG. **10** is a schematic diagram depicting an LDR/PCR process for multiplex detection of gene amplifications and deletions. Here, the ratio of the Her-2/neu gene from Chromosome 17q, the p53 gene from Chromosome 17p, and the SOD gene from Chromosome 21q is detected. Following denaturation of DNA at 94° C., pairs of oligonucleotide probes, having a target-specific portion and a primer-specific portion, are allowed to anneal adjacent to each other on target nucleic acids and ligate to one another (in the absence of mismatches). This ligase detection reaction is carried out with Tth ligase at a hybridization/ligation temperature of 65° C. which is well below the T_m values of 75° C. for the oligonucleotide probes. Next, the ligation product sequences are simultaneously amplified by PCR using Taq polymerase and two common primers complementary to the primer-specific portion, one of which is fluorescently labeled. This maintains the proportionality of the target sequences initially present in the sample. The extension products are then digested with HaeIII and HinplI which releases fluorescently labeled fragments of unique sizes for each target sequence present in the sample. The digestion products are separated and analyzed on an Applied Biosystems, Inc. (Foster City, Calif.) 373A DNA Sequencer. The peak heights and areas are related to the relative copies of genes present in the initial target sample.

FIG. **11** is a schematic diagram, depicting a problem which can be encountered with the allele-specific LDR/PCR process. While a PCR/LDR process is very powerful, there may be circumstances where a multiplexed allele-specific LDR/PCR process of the present invention would be preferred. The concept is to have one or more sets of LDR oligonucleotide probes, each set characterized by (a) a first oligonucleotide probe, having a target-specific portion and a 5' upstream primer-specific portion, and (b) a second oligonucleotide probe, having a target-specific portion and a 3' downstream primer-specific portion. As shown in step **1** of FIG. **11**, the LDR oligonucleotide probes anneal adjacent to each other on the target sequence. An LDR reaction using thermostable ligase (black dot) would form a ligation product sequence provided there is perfect complementarity at the ligation junction. In step **2**, the ligation product sequences are PCR amplified with primer sets, each set characterized by (a) an upstream primer containing the same sequence as the 5' upstream primer-specific portion of a ligation product sequence and (b) a downstream primer complementary to the 3' downstream primer-specific portion of that ligation product sequence. The primers are shown as black lines in step **2**. If one primer is fluorescently labeled, it will generate a fluorescent product which may be detected in a variety of detection schemes. For the LDR/PCR process to be specific, a PCR extension product should not be formed in the absence of a ligation event. Unfortunately, the possibility exists for polymerase to extend the first LDR oligonucleotide probe (off normal target), forming a product containing the length of the target sequence, and a primer-specific portion on the 5' end. Meanwhile, polymerase can make several complementary copies of the downstream LDR probe using the downstream primer. In a second amplification cycle, this downstream LDR probe extension product can anneal to the upstream LDR probe extension product off the target sequence, and generate a sequence containing the target region flanked by the two primer-specific sequences. This product will amplify as the LDR product, and thus yield a false positive signal.

FIG. **12** is a schematic drawing showing a solution to the allele specific LDR/PCR problem, utilizing an intermediate exonuclease digestion step. Allele-specific LDR/PCR can be achieved while significantly reducing background ligation independent (incorrect) target amplification. To do so, it is necessary to eliminate one or more of the components required for ligation independent PCR amplification, without removing the information content of the ligation product sequence. One solution is to use exonuclease in step **2** to digest unreacted LDR oligonucleotide probes from step **1**. By blocking the end which is not ligated, for example the 3' end of the downstream oligonucleotide probe, one probe can be made substantially resistant to digestion, while the other is sensitive. Only the presence of full length ligation product sequence will prevent digestion of the upstream primer. Blocking groups include use of a thiophosphate group and/or use of 2-O-methyl ribose sugar groups in the backbone. Exonucleases include Exo 1 (3'-5'), Exo III (3'-5'), and Exo IV (both 5'-3' and 3'-5'), the later requiring blocking on both sides. One convenient way to block both probes is by using one long "padlock" probe (see M. Nilsson et. al., "Padlock Probes: Circularizing Oligonucleotides for Localized DNA Detection," *Science* 265: 2085-88 (1994), which is hereby incorporated by reference), although this is by no means required. An advantage of using exonucleases for example a combination of Exo I (single strand specific) and Exo III (double strand specific), is the ability to destroy both target and one LDR probe, while leaving the ligation product sequences substantially undigested. By using an exonuclease treatment prior to PCR, in accordance with steps 3 and 4, either one or both oligonucleotide probes in each set are substantially reduced, and thus hybridization of the remaining oligonucleotide probes to the original target DNA (which is also substantially reduced by exonuclease treatment) and formation of a ligation product sequence which is a suitable substrate for PCR amplification by the oligonucleotide primer set is substantially reduced. In other words, formation of ligation independent labeled extension products is substantially reduced or eliminated.

FIG. **13** is a flow diagram showing an allele-specific LDR/PCR process using exonuclease digestion with either size based- or DNA array based-detection. The flow diagram shows the three reactions required for the multiplexed

allele-specific LDR/PCR process. In step **1**, sets of LDR oligonucleotide probes (wherein the downstream probes are blocked on their 3' ends) are ligated in the presence of the correct allele target using Taq DNA ligase. Unreacted upstream probes are digested with exonuclease in step **2**, coincidentally, target is also digested. Finally, in step **3**, primer sets are used to amplify the ligation product sequence by hybridizing to the primer specific portions of ligation product sequences. In step **4***a*, the LDR oligonucleotide probes in a given particular set generate a unique length product, and thus may be distinguished from either oligonucleotide probes or other ligation products. After the PCR reaction, the products are separated by size or electrophoretic mobility. Labels on the PCR primers are detected, and the products are distinguished by size. In step **4***b*, the LDR oligonucleotide probes in a particular set use may be distinguished from either oligonucleotide probes or other ligation product sequences by differences in the sequences of the PCR primers. By using a plurality of oligonucleotide primer sets, each set characterized by (a) an upstream primer containing the same sequence as the 5' upstream primer-specific portion of a ligation product sequence, and (b) a downstream primer complementary to the 3' downstream primer-specific portion of that ligation product sequence, wherein one primer has a detectable reporter label and the other primer contains an addressable nucleotide sequence linked to the 5' end of that primer such that the addressable nucleotide sequence remains single stranded after a PCR reaction, all the products can be distinguished. The latter may be achieved by using a non-natural base within a PCR primer which polymerase cannot extend through, thus generating PCR products which have single stranded tails. See C. Newton, et. al., "The Production of PCR Products with 5' Single-stranded Tails Using Primers that Incorporate Novel Phosphoramidite Intermediates," *Nucl. Acids Res.* 21(3): 1155-62 (1993), which is hereby incorporated by reference. By providing a DNA array with different capture oligonucleotides immobilized at different particular sites, where the capture oligonucleotides have nucleotide sequences complementary to the addressable nucleotide sequences on the primers, the PCR extension products can hybridize to the DNA array. Finally, the labels of extension product sequences captured using the addressable array-specific portions immobilized to the DNA array at particular sites can be detected. This indicates the presence of one or more target nucleotide sequences in the sample.

FIG. **14** is a flow diagram showing a quantitative allele-specific LDR/PCR process using exonuclease digestion in step **3** with either size based- or DNA array based-detection. The flow diagram shows how one can quantify the amounts of different targets (especially low abundance cancer mutations) by adding marker sequence(s) (step **1**) at the start of the LDR reaction (step **2**). In this embodiment, the biochemical reactions (i.e. PCR (step **4**)) are followed, as described in FIG. **13**, and the relative amount of mutant product to marker product are quantified using capillary or gel electrophoresis (step **5***a*) or capture on an addressable array (step **5***b*). The amount of mutant target present in the original sample can then be determined.

FIG. **15** is a schematic drawing showing an allele-specific LDR/PCR process with exonuclease digestion (step **2**) for detection of mutations or polymorphisms. Mutations and polymorphisms may be distinguished as described in FIG. **12**. In this example, in step **1**, the upstream LDR oligonucleotide probes, which have the discriminating allele-specific base at the 3' end of the target-specific portion, have different 5' upstream primer-specific portions. Thus, different primers (in the PCR amplification step (i.e. step **3**)) may be labeled with different fluorescent groups (Fam and Tet) to allow for distinction of products (step **4**). An array based detection scheme may also be used, where the upstream (allele-specific) probes have different 5' upstream primer-specific portions, and the different PCR primers contain different addressable nucleotide sequences which remain single stranded after a PCR reaction.

FIG. **16** is a schematic drawing showing an allele-specific LDR (step 1)/PCR (step **3**) process using exonuclease digestion (step **2**) for detection of mononucleotide or dinucleotide repeat polymorphisms. One of the most powerful uses of LDR/PCR is for detecting nucleotide repeat polymorphisms, a task which cannot be achieved by allele-specific PCR (because the 3' nucleotide is always the same), nor easily achieved by observing PCR product size variation (due to Taq polymerase slippage during amplification). In FIG. **16**, the LDR oligonucleotide probes distinguish between an A_9 and A_{10} mononucleotide repeat sequence by virtue of the specificity of thermostable DNA ligase. LDR products are only formed on the correct length target sequence, and thus the presence of that target is distinguished (step **4**).

FIG. **17** is schematic drawing showing an allele-specific LDR/PCR process using exonuclease digestion (step **2**) for detection of low abundance mononucleotide or dinucleotide repeat mutations. Mononucleotide repeat length mutations may be distinguished as described in FIG. **12**. In FIG. **17**, the LDR oligonucleotide probes (step **1**) distinguish between an A_8, A_9 (mutants), and A_{10} (normal) mononucleotide repeat sequences by virtue of the specificity of thermostable DNA ligase. The two upstream LDR oligonucleotide probes differ in the length of the mononucleotide sequence at their 3' ends of their target specific portion and have different 5' upstream primer-specific portions. Thus, different primers (in the PCR amplification step (step **3**)) may be labeled with different fluorescent groups (Fam and Tet) to allow for distinction of products (step **4**). This has the distinct advantage of allowing mononucleotide repeat polymorphisms to be distinguished based on fluorescent label instead of size, the latter being susceptible to false positives due to polymerase slippage. An array based detection scheme may also be used, where the upstream (allele-specific) probes have different 5' upstream primer-specific portions, and the different PCR primers contain different addressable nucleotide sequences which remain single stranded after a PCR reaction.

FIG. **18** is a flow diagram, showing an allele-specific LDR/PCR process using uracil. N-glycosylase selection with either size based- or DNA array based-detection. The flow diagram shows the four reactions required for multiplexed allele-specific LDR/PCR. Sets of LDR oligonucleotide probes (wherein one or both probes contain deoxyuracil in place of deoxythimidine) are ligated in the presence of the correct allele target using Taq DNA ligase in step **1**. A complementary copy of the ligation product sequence is made with sequenase in step **2**. Sequenase is a modified T7 polymerase, with any easily inactivated polymerase (i.e. mesophilic polymerases such as, *E. coli* polymerase) being useful. Both ligation product sequences and unreacted probes are destroyed with uracil N-glycosylase in step **3**. The advantage of using uracil N-glycosylase is its proven ability in carry-over prevention for PCR. Finally PCR primer sets are used to amplify the sequenase extension products in step **4**. In step **5***a*, the LDR oligonucleotide probes in a particular set generate a unique length product, and thus may be distinguished from either probes or other ligation products. After the PCR reaction, the products are separated by size or electrophoretic mobility. Labels on the

PCR primers are detected, and products are distinguished by size. In step **5***b*, the LDR oligonucleotide probes in a particular set may be distinguished from either probes or other ligation product sequences by differences in the sequences of the primer-specific portions. By using a plurality of oligonucleotide primer sets, each set characterized by (a) an upstream primer containing the same sequence as the 5' upstream primer-specific portion of a ligation product sequence, and (b) a downstream primer complementary to the 3' downstream primer-specific portion of that ligation product sequence. One primer has a detectable reporter label, and the other primer contains an addressable array-specific portion linked to the 5' end of that primer such that the addressable array-specific portion remains single stranded after a PCR reaction, one can distinguish all the products. By providing a DNA array with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to the addressable array-specific portions on the primers, the PCR extension products can be hybridized to the DNA array. Finally, the labels of extension product sequences captured using the addressable nucleotide sequence portions and immobilized to the DNA array at particular sites can be detected to indicate the presence of one or more target nucleotide sequences in the sample.

FIG. **19** is a flow diagram showing a quantitative allele-specific LDR/PCR process using uracil N-glycosylase selection with either size based- or DNA array based-detection. The flow diagram shows how one can quantify the amounts of different targets (especially low abundance cancer mutations) by adding marker sequence(s) in step **1** at the start of the LDR phase in step **2**. As described in FIG. **18**, the biochemical reactions (i.e. sequenase treatment (step **3**), uracil N-glycosylase selection (step **4**), and PCR (step **5**)) are preceded with, and the relative amount of mutant product to marker product is quantified using capillary or gel electrophoresis (step **6***a*) or capture on an addressable array (step **6***b*). From this information, the amount of mutant target present in the original sample can be determined.

FIG. **20** is a schematic drawing showing an allele-specific LDR/PCR process using uracil N-glycosylase selection (step **3**) (after sequenase treatment (step **2**)) for detection of mononucleotide or dinucleotide repeat polymorphisms. One of the most powerful uses of the LDR/PCR process is for detecting nucleotide repeat polymorphisms, a task which cannot be achieved by allele-specific PCR (since the 3' nucleotide is always the same), nor easily achieved by observing PCR product size variation (due to Taq polymerase slippage during amplification), as in step **4**. In FIG. **20**, the LDR (step **1**) oligonucleotide probes distinguish between an A_9 and A_{10} mononucleotide repeat sequence by virtue of the specificity of thermostable DNA ligase. Ligation product sequences are only formed on the correct length target sequence, and, thus, the presence of that target is distinguished in step **5**.

FIG. **21** is a schematic drawing showing an allele-specific LDR/PCR process using uracil N-glycosylase selection for detection of low abundance mononucleotide or dinucleotide repeat mutations. Mononucleotide repeat length mutations may be distinguished as described in FIG. **18**. In FIG. **21**, the LDR oligonucleotide probes distinguish between an A_8, A_9 (mutants), and A_{10} (normal) mononucleotide repeat sequences by virtue of the specificity of thermostable DNA ligase (step **1**). Sequenase treatment (step **2**) and uracil N-glycosylase selection (step 3) are then carried out. The two upstream LDR oligonucleotide probes differ in the length of the mononucleotide sequence at the 3' ends of their target-specific portion, and have different 5' upstream primer-specific portions. Thus, different primers (in the PCR amplification step (steps 4-5)) may be labeled with different fluorescent groups (Fam and Tet) to allow for distinction of products. This has the distinct advantage of allowing one to distinguish mononucleotide repeat polymorphisms based on fluorescent label instead of size, the latter being susceptible to false positives due to polymerase slippage. An array based detection scheme may also be used, where the upstream (allele-specific) probes have different 5' upstream primer-specific portions, and the different PCR primers contain different addressable array-specific portions which remain single stranded after a PCR reaction.

The LDR/exonuclease/PCR process described with reference to FIGS. **11** to **17** and the LDR/sequenase/uracil N-glycosylase/PCR process set forth in FIGS. **18-21** provide the ability to multiplex detect and then PCR amplify many different target sequences and to distinguish multiple single-base or sequence variations, all in a single reaction tube. This is achieved by combining the sensitivity of PCR with the selectivity of LDR. Since the selection of mutant sequences is mediated by LDR rather than PCR, the primary PCR/secondary PCR/LDR process is less susceptible to false-positive signal generation. In addition, the primary PCR/secondary PCR/LDR process allows detection of closely-clustered mutations, detection of single base or small insertions and deletions in small repeat sequences, quantitative detection of less than 1% mutations in high background of normal DNA, and detection of ligation product sequences using addressable arrays. Detection of single base or small insertions and deletions in small and medium repeat sequences may cause "stutter" when the primary amplification is PCR. No other currently-available technique can adequately solve this problem, especially when the target sequence containing the mononucleotide repeat polymorphism is present in a lower abundance than normal DNA. In fact, analysis of genomic mutations which involve repeat sequence changes is severely hampered by the PCR "stutter" problem. By using the LDR/PCR process of the present invention, it is possible to detect down to 1% mutations in a high background of normal DNA. The only relatively minor challenges presented by this process are that the mutations must be known and that 3 different enzymes/reaction conditions must be utilized.

III. Primary PCR/Secondary PCR Process

A third aspect of the present invention also involves a method for identifying two or more of a plurality of sequences differing by one or more single-base changes, insertions, deletions, or translocations in one or more target nucleotide sequences. This method involves subjecting a sample potentially containing one or more target nucleotide sequences with a plurality of sequence differences to two successive polymerase chain reaction phases.

For the first polymerase chain reaction phase, one or more primary oligonucleotide primer groups are provided where each group comprises one or more primary oligonucleotide primer sets. Each set has a first oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primary-specific portion, and a second oligonucleotide primer, having a target-specific portion and a 5' upstream secondary primer-specific portion. The first oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion and the second oligonucleotide primers of each set in the same group contain the same 5' upstream secondary primer-specific portion. The oligonucleotide primers in a particular set are

suitable for hybridization on complementary strands of a corresponding target nucleotide sequence to permit formation of a polymerase chain reaction product. However, there is a mismatch which interferes with formation of such a polymerase chain reaction product when the primary oligonucleotide primers hybridize to any other nucleotide sequence present in the sample. The polymerase chain reaction products in a particular set may be distinguished from other polymerase chain reaction products with the same group or other groups. The primary oligonucleotide primers are blended with the sample and the polymerase to form a primary polymerase chain reaction mixture.

The primary polymerase chain reaction mixture is subjected to two or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as described above. During the hybridization treatment, the target-specific portion of a primary oligonucleotide primer is hybridized to the target nucleotide sequences. In the extension treatment, the hybridized primary oligonucleotide primers are extended to form primary extension products complementary to the target nucleotide sequence to which the primary oligonucleotide primer is hybridized.

Although the upstream secondary primer-specific portions of a primary oligonucleotide primer set are not present on the target DNA, their sequences are copied by the second and subsequent cycles of the primary polymerase chain reaction phase. As a result, the primary extension products produced after the second cycle have the secondary primer-specific portions on their 5' ends and the complement of primer-specific portion on their 3' ends.

In the second polymerase chain reaction phase of this aspect of the present invention, one or a plurality of secondary oligonucleotide primer sets are provided. Each set has a first secondary primer having a detectable reporter label and containing the same sequence as the 5' upstream portion of a first primary oligonucleotide primer, and a second secondary primer containing the same sequence as the 5' upstream primer of the second primary oligonucleotide primer from the same primary oligonucleotide primer set as the first primary oligonucleotide complementary to the first secondary primer. A set of secondary oligonucleotide primers amplify the primary extension products in a given group. The secondary oligonucleotide primers are blended with the primary extension products and the polymerase to form a secondary polymerase chain reaction mixture.

The secondary polymerase chain reaction mixture is subjected to one or more polymerase chain reaction cycles involving a denaturation treatment, a hybridization treatment, and an extension treatment, as described above. In the hybridization treatment, the secondary oligonucleotide primers are hybridized to the primary extension products, while the extension treatment causes the hybridized secondary oligonucleotide primers to be extended to form secondary extension products complementary to the primary extension products. After subjecting the secondary polymerase chain reaction mixture to the two or more polymerase chain reaction cycles, the labelled secondary extension products are detected. This indicates the presence of one or more target nucleotide sequences in the sample.

FIG. **22** is a flow diagram depicting a primary PCR/secondary PCR process, in accordance with the present invention, for detection of microsatellite repeats. In step **1** (i.e. the primary PCR phase), after DNA sample preparation, multiple exons are amplified using Taq polymerase under hot start conditions with oligonucleotide primers having a target-specific portion and a secondary primer-specific portion. step **2** involves a secondary PCR phase where Taq polymerase is used to amplify the primary PCR extension products with oligonucleotide primers containing the same sequence as the secondary primer-specific portion of the primary PCR primers. The extension products resulting from the secondary PCR phase are subjected in step **3** to capillary electrophoresis or gel electrophoresis, followed by fluorescent quantification. The electrophoresis results in FIG. **22** indicate the presence of both alleles (i.e., chromosomes) containing RB1 and NM23 and loss of heterozygosity (i.e., loss of allele on one chromosome) for p53.

FIG. **23** is a schematic diagram depicting a primary PCR/secondary PCR process, according to the present invention, for detection of the loss of heterozygosity due to insertions and deletions in microsatellite repeats. The primary PCR phase in step **1** is initiated by denaturing the sample DNA at 94° C. Long PCR oligonucleotide primers, having 3' ends complementary to unique DNA surrounding microsatellite repeat sequences and 5' ends containing the same sequence as one of two primers utilized in the secondary PCR phase, are then caused to anneal to target DNA at 65° C. The primary PCR phase is carried out for 10-15 cycles. The long primers utilized in the primary PCR phase can be multiplexed as long as they do not amplify alleles with overlapping length ranges. These reactions must be carried out on tumor and corresponding normal DNA to identify informative (i.e heterozygous) loci. In step **2** (i.e secondary PCR amplification), primers complementary to the 5' ends of the primary PCR primers (one fluorescently labeled) are then used to amplify the primary PCR extension products at nearly equal efficiency. The secondary PCR extension products are then separated and analyzed by gel electrophoresis and an Applied Biosystems Inc. 373A DNA Sequencer using the Genescan 672 software package. Areas of loss of heterozygosity at informative loci are identified. The analysis in FIG. **23** shows the presence of both alleles (i.e., chromosomes) containing RB1 and NM23 and loss of heterozygosity (i.e., loss of allele on one chromosome) for p53.

IV. General Process Information

The ligase detection reaction is described generally in WO 90/17239 to Barany et al., F. Barany et al., "Cloning, Overexpression and Nucleotide Sequence of a Thermostable DNA Ligase-encoding Gene," *Gene* 109:1-11 (1991), and F. Barany, "Genetic Disease Detection and DNA Amplification Using Cloned Thermostable Ligase," *Proc. Natl. Acad. Sci. USA*, 88:189-193 (1991), the disclosures of which are hereby incorporated by reference. In accordance with the present invention, the ligase detection reaction can use 2 sets of complementary oligonucleotides. This is known as the ligase chain reaction which is described in the 3 immediately preceding references, which are hereby incorporated by reference. Alternatively, the ligase detection reaction can involve a single cycle which is known as the oligonucleotide ligation assay. See Landegren, et al., "A Ligase-Mediated Gene Detection Technique," *Science* 241:1077-80 (1988); Landegren, et al.; "DNA Diagnostics—Molecular Techniques and Automation," *Science* 242:229-37 (1988); and U.S. Pat. No. 4,988,617 to Landegren, et al., which are hereby incorporated by reference

During ligase detection reaction phases, the denaturation treatment is carried out at a temperature of 80-105° C., while hybridization takes place at 50-85° C. Each cycle comprises a denaturation treatment and a thermal hybridization treatment which in total is from about one to five minutes long. Typically, the ligation detection reaction involves repeatedly

denaturing and hybridizing for 2 to 50 cycles. The total time for the ligase detection reaction phase is 1 to 250 minutes.

The oligonucleotide probe sets or primers can be in the form of ribonucleotides, deoxynucleotides, modified ribonucleotides, modified deoxyribonucleotides, modified phosphate-sugar-backbone oligonucleotides, nucleotide analogs, and mixtures thereof.

In one variation, the oligonucleotides of the oligonucleotide probe sets each have a hybridization or melting temperature (i.e. T_m) of 66-70° C. These oligonucleotides are 20-28 nucleotides long.

The oligonucleotide probe sets or primers, as noted above, have a reporter label suitable for detection. Useful labels include chromophores, fluorescent moieties, enzymes, antigens, heavy metals, magnetic probes, dyes, phosphorescent groups, radioactive materials, chemiluminescent moieties, and electrochemical detecting moieties.

The polymerase chain reaction process is fully described in H. Erlich, et. al., "Recent Advances in the Polymerase Chain Reaction," *Science* 252: 1643-50 (1991); M. Innis, et. al., *PCR Protocols: A Guide to Methods and Applications*, Academic Press: New York (1990); and R. Saiki, et. al., "Primer-directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase," *Science* 239: 487-91 (1988), which are hereby incorporated by reference.

A particularly important aspect of the present invention is its capability to quantify the amount of target nucleotide sequence in a sample. This can be achieved in a number of ways by establishing standards which can be internal (i.e. where the standard establishing material is amplified and detected with the sample) or external (i.e. where the standard establishing material is not amplified, and is detected with the sample).

In accordance with one quantification method, the signal generated by the reporter label, resulting from capture of ligation product sequences produced from the sample being analyzed, are detected. The strength of this signal is compared to a calibration curve produced from signals generated by capture of ligation product sequences in samples with known amounts of target nucleotide sequence. As a result, the amount of target nucleotide sequence in the sample being analyzed can be determined. This techniques involves use of an external standard.

Another quantification method, in accordance with the present invention, relates to an internal standard. Here, a known amount of one or more marker target nucleotide sequences is added to the sample. In addition, a plurality of marker-specific oligonucleotide probe sets are added along with the ligase, the previously-discussed oligonucleotide probe sets, and the sample to a mixture. The marker-specific oligonucleotide probe sets have (1) a first oligonucleotide probe with a target-specific portion complementary to the marker target nucleotide sequence, and (2) a second oligonucleotide probe with a target-specific portion complementary to the marker target nucleotide sequence and a detectable reporter label. The oligonucleotide probes in a particular marker-specific oligonucleotide set are suitable for ligation together when hybridized adjacent to one another on a corresponding marker target nucleotide sequence. However, there is a mismatch which interferes with such ligation when hybridized to any other nucleotide sequence present in the sample or added marker sequences. The presence of ligation product sequences is identified by detection of reporter labels. The amount of target nucleotide sequences in the sample is then determined by comparing the amount of ligation product sequence generated from known amounts of marker target nucleotide sequences with the amount of other ligation product sequences.

Another quantification method, in accordance with the present invention involves, analysis of a sample containing two or more of a plurality of target nucleotide sequences with a plurality of sequence differences. Here, ligation product sequences corresponding to the target nucleotide sequences are detected and distinguished by any of the previously-discussed techniques. The relative amounts of the target nucleotide sequences in the sample are then quantified by comparing the relative amounts of captured ligation product sequences generated. This provides a quantitative measure of the relative level of the target nucleotide sequences in the sample.

The preferred thermostable ligase is that derived from *Thermus aquaticus*. This enzyme can be isolated from that organism. M. Takahashi, et al., "Thermophillic DNA Ligase," *J. Biol. Chem.* 259:10041-47 (1984), which is hereby incorporated by reference. Alternatively, it can be prepared recombinantly. Procedures for such isolation as well as the recombinant production of *Thermus aquaticus* ligase (as well as *Thermus themophilus* ligase) are disclosed in WO 90/17239 to Barany, et. al., and F. Barany, et al., "Cloning, Overexpression and Nucleotide Sequence of a Thermostable DNA-Ligase Encoding Gene," Gene 109:1-11 (1991), which are hereby incorporated by reference. These references contain complete sequence information for this ligase as well as the encoding DNA. Other suitable ligases include *E. coli* ligase, T4 ligase, and *Pyococcus* ligase.

The ligation detection reaction mixture may include a carrier DNA, such as salmon sperm DNA.

The hybridization step in the ligase detection reaction, which is preferably a thermal hybridization treatment discriminates between nucleotide sequences based on a distinguishing nucleotide at the ligation junctions. The difference between the target nucleotide sequences can be, for example, a single nucleic acid base difference, a nucleic acid deletion, a nucleic acid insertion, or rearrangement. Such sequence differences involving more than one base can also be detected. Preferably, the oligonucleotide probe sets have substantially the same length so that they hybridize to target nucleotide sequences at substantially similar hybridization conditions. As a result, the process of the present invention is able to detect infectious diseases, genetic diseases, and cancer. It is also useful in environmental monitoring, forensics, and food science.

A wide variety of infectious diseases can be detected by the process of the present invention. Typically, these are caused by bacterial, viral, parasite, and fungal infectious agents. The resistance of various infectious agents to drugs can also be determined using the present invention.

Bacterial infectious agents which can be detected by the present invention include *Escherichia coli, Salmonella, Shigella, Klebsiella, Pseudomonas, Listeria monocytogenes, Mycobacterium tuberculosis, Mycobacterium avium-intracellulare, Yersinia, Francisella, Pasteurella, Brucella, Clostridia, Bordetella pertussis, Bacteroides, Staphylococcus aureus, Streptococcus pneumonia, B-Hemolytic strep., Corynebacteria, Legionella, Mycoplasma, Ureaplasma, Chlamydia, Neisseria gonorrhea, Neisseria meningitides, Hemophilus influenza, Enterococcus faecalis, Proteus vulgaris, Proteus mirabilis, Helicobacter pylori, Treponema palladium, Borrelia burgdorferi, Borrelia recurrentis, Rickettsial pathogens, Nocardia*, and *Acitnomycetes.*

Fungal infectious agents which can be detected by the present invention include *Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidio-*

ides immitis, Paracoccidioides brasiliensis, Candida albicans, Aspergillus fumigautus, Phycomycetes (Rhizopus), *Sporothrix schenckii, Chromomycosis*, and *Maduromycosis.*

Viral infectious agents which can be detected by the present invention include human immunodeficiency virus, human T-cell lymphocytotrophic virus, hepatitis viruses (e.g., Hepatitis B Virus and Hepatitis C Virus), Epstein-Barr Virus, cytomegalovirus, human papillomaviruses, orthomyxo viruses, paramyxo viruses, adenoviruses, corona viruses, rhabdo viruses, polio viruses, toga viruses, bunya viruses, arena viruses, rubella viruses, and reo viruses.

Parasitic agents which can be detected by the present invention include *Plasmodium falciparum, Plasmodium malaria, Plasmodium vivax, Plasmodium ovale, Onchoverva volvulus, Leishmania, Trypanosoma* spp., *Schistosoma* spp., *Entamoeba histolytica, Cryptosporidum, Giardia* spp., *Trichimonas* spp., *Balatidium coli, Wuchereria bancrofti, Toxoplasma* spp., *Enterobius vermicularis, Ascaris lumbricoides, Trichuris trichiura, Dracunculus medinesis, trematodes, Diphyllobothrium latum, Taenia* spp., *Pneumocystis carinii*, and *Necator americanis.*

The present invention is also useful for detection of drug resistance by infectious agents. For example, vancomycin-resistant *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus*, penicillin-resistant *Streptococcus pneumoniae*, multi-drug resistant *Mycobacterium tuberculosis*, and AZT-resistant human immunodeficiency virus can all be identified with the present invention.

Genetic diseases can also be detected by the process of the present invention. This can be carried out by prenatal or post-natal screening for chromosomal and genetic aberrations or for genetic diseases. Examples of detectable genetic diseases include: 21 hydroxylase deficiency, cystic fibrosis, Fragile X Syndrome, Turner Syndrome, Duchenne Muscular Dystrophy, Down Syndrome or other trisomies, heart disease, single gene diseases, HLA typing, phenylketonuria, sickle cell anemia, Tay-Sachs Disease, thalassemia, Klinefelter Syndrome, Huntington Disease, autoimmune diseases, lipidosis, obesity defects, hemophilia, inborn errors of metabolism, and diabetes.

Cancers which can be detected by the process of the present invention generally involve oncogenes, tumor suppressor genes, or genes involved in DNA amplification, replication, recombination, or repair. Examples of these include: BRCA1 gene, p53 gene, APC gene, Her2/Neu amplification, Bcr/Abl, K-ras-gene, and human papillomavirus Types 16 and 18. Various aspects of the present invention can be used to identify amplifications, large deletions as well as point mutations and small deletions/insertions of the above genes in the following common human cancers: leukemia, colon cancer, breast cancer, lung cancer, prostate cancer, brain tumors, central nervous system tumors, bladder tumors, melanomas, liver cancer, osteosarcoma and other bone cancers, testicular and ovarian carcinomas, head and neck tumors, and cervical neoplasms.

In the area of environmental monitoring, the present invention can be used for detection, identification, and monitoring of pathogenic and indigenous microorganisms in natural and engineered ecosystems and microcosms such as in municipal waste water purification systems and water reservoirs or in polluted areas undergoing bioremediation. It is also possible to detect plasmids containing genes that can metabolize xenobiotics, to monitor specific target microorganisms in population dynamic studies, or either to detect, identify, or monitor genetically modified microorganisms in the environment and in industrial plants.

The present invention can also be used in a variety of forensic areas, including for human identification for military personnel and criminal investigation, paternity testing and family relation analysis, HLA compatibility typing, and screening blood, sperm, or transplantation organs for contamination.

In the food and feed industry, the present invention has a wide variety of applications. For example, it can be used for identification and characterization of production organisms such as yeast for production of beer, wine, cheese, yogurt, bread, etc. Another area of use is with regard to quality control and certification of products and processes (e.g., livestock, pasteurization, and meat processing) for contaminants. Other uses include the characterization of plants, bulbs, and seeds for breeding purposes, identification of the presence of plant-specific pathogens, and detection and identification of veterinary infections.

Desirably, the oligonucleotide probes are suitable for ligation together at a ligation junction when hybridized adjacent to one another on a corresponding target nucleotide sequence due to perfect complementarity at the ligation junction. However, when the oligonucleotide probes in the set are hybridized to any other nucleotide sequence present in the sample, there is a mismatch at a base at the ligation junction which interferes with ligation. Most preferably, the mismatch is at the base adjacent the 3' base at the ligation junction. Alternatively, the mismatch can be at the bases adjacent to bases at the ligation junction.

As noted supra, detection and quantification can be carried out using capillary or gel electrophoresis or on a solid support with an array capture oligonucleotides.

The use of capillary and gel electrophoresis for such purposes is well known. See e.g., Grossman, et. al., "High-density Multiplex Detection of Nucleic Acid Sequences: Oligonucleotide Ligation Assay and Sequence-coded Separation," *Nucl. Acids Res.* 22(21): 4527-34 (1994), which is hereby incorporated by reference.

The use of a solid support with an array of capture oligonucleotides is fully disclosed in pending provisional U.S. patent application Ser. No. 60/011,359, which is hereby incorporated by reference. When using such arrays, the oligonucleotide primers or probes used in the above-described coupled PCR and LDR phases, respectively, have an addressable array-specific portion. After the LDR or PCR phases are completed, the addressable array-specific portions for the products of such processes remain single stranded and are caused to hybridize to the capture oligonucleotides during a capture phase. C. Newton, et al., "The Production of PCR Products With 5' Single-Stranded Tails Using Primers That Incorporate Novel Phosphoramidite Intermediates," *Nucl. Acids Res.* 21(5):1155-62 (1993), which is hereby incorporated by reference.

During the capture phase of the process, the mixture is contacted with the solid support at a temperature of 45-90° C. and for a time period of up to 60 minutes. Hybridizations may be accelerated by adding cations, volume exclusion or chaotropic agents. When an array consists of dozens to hundreds of addresses, it is important that the correct ligation product sequences have an opportunity to hybridize to the appropriate address. This may be achieved by the thermal motion of oligonucleotides at the high temperatures used, by mechanical movement of the fluid in contact with the array surface, or by moving the oligonucleotides across the array by electric fields. After hybridization, the array is washed sequentially with a low stringency wash buffer and then a high stringency wash buffer.

It is important to select capture oligonucleotides and addressable nucleotide sequences which will hybridize in a stable fashion. This requires that the oligonucleotide sets and the capture oligonucleotides be configured so that the oligonucleotide sets hybridize to the target nucleotide sequences at a temperature less than that which the capture oligonucleotides hybridize to the addressable array-specific portions. Unless the oligonucleotides are designed in this fashion, false positive signals may result due to capture of adjacent unreacted oligonucleotides from the same oligonucleotide set which are hybridized to the target.

The capture oligonucleotides can be in the form of ribonucleotides, deoxyribonucleotides, modified ribonucleotides, modified deoxyribonucleotides, peptide nucleotide analogues, modified peptide nucleotide analogues, modified phosphate-sugar backbone oligonucleotides, nucleotide analogues, and mixtures thereof.

Where an array is utilized, the detection phase of the process involves scanning and identifying if LDR or PCR products have been produced and correlating the presence of such products to a presence or absence of the target nucleotide sequence in the test sample. Scanning can be carried out by scanning electron microscopy, confocal microscopy, charge-coupled device, scanning tunneling electron microscopy, infrared microscopy, atomic force microscopy, electrical conductance, and fluorescent or phosphor imaging. Correlating is carried out with a computer.

EXAMPLES

LDR/PCR Process

Example 1

Genomic DNA Preparation

Genomic DNA was prepared from the blood of two normal human volunteers, one male and one female, according to standard techniques. Briefly, approximately 12 ml of blood was obtained in EDTA-containing blood collection tubes. Red blood cells were lysed by mixing the blood samples with 4 volumes of lysis buffer (10 mM Tris pH 8.0, 10 mM EDTA). After 10 min on ice with occasional agitation, the suspensions were centrifuged and the supernatants were decanted. The white blood cell pellets were resuspended in 20 ml of lysis buffer, and the above process was repeated. Each cell pellet was then suspended in 15 ml of digestion buffer (50 mM Tris pH 8.0, 5 mM EDTA, 100 mM NaCl, 1% SDS) and 3 mg (0.2 mg/ml) of proteinase K was added. The cells were digested at 37° C. for 5 hours. The digests were extracted twice with equal volumes of phenol, then once with equal volumes of a 1:1 phenol:chloroform mixture and finally once with equal volumes of chloroform, each time centrifuging the mixture and removing the aqueous phase for the next extraction. After the final extraction and removing the aqueous phases, one tenth volume of 3 M sodium acetate, pH 6.5, was added. Two volumes of ice cold 100% EtOH were then added to each solution to precipitate the genomic DNAs, which were spooled out of solution on glass pipettes. The DNA precipitates were washed twice in 0.75 ml volumes of 70% EtOH, briefly centrifuging each time to allow removal of the supernatants. After removing the supernatants for the second time, the remaining EtOH was allowed to evaporate and the DNA was suspended in 0.5 ml of TE (10 mM Tri-HCl pH 8.0 containing 1 mM EDTA) solution. A fifth dilution of each DNA solution was also prepared in TE.

To determine the concentrations of the one fifth DNA solutions, 1, 2, and 4 μl aliquots of each were loaded on a 1% agarose gel with a known amount of HindIII digested lambda DNA as a control. The gel was run at 150 Volts for 2 hours with ethidium bromide in the electrophoresis buffer. After photographing the gel and comparing the intensities of the DNA bands, the one fifth dilutions were judged to have concentrations of approximately 100 ng/ml. DNA solutions extracted from various tumor cell lines were the generous gifts of other laboratories. The concentrations of these solutions were checked in a similar fashion and solutions of 100 ng/ml in TE were prepared.

To digest the genomic DNAs with Taq I, 25 μl of the 100 ng/μl solutions was mixed with 5 μl of 10× medium salt buffer (0.5 M NaCl, 0.1 M $MgCl_2$, 0.1 M Tris, pH 8.0), 20 μl of water-ME (i.e. water containing 6 mM ME (i.e., mercaptoethanol)), and 400 U of Taq I restriction endonuclease. The digests were covered with mineral oil and incubated at 65° C. for 1 hour. The reactions were stopped by adding 1.2 μl of 500 mM EDTA and heating the specimens to 85° C. for 10 min. Complete digestion of the DNAs was checked by electrophoresing aliquots on a 1% agarose gel.

Example 2

Oligonucleotide Preparation for LDR Probes and PCR Primers

All oligonucleotides were synthesized on a 394A DNA Synthesizer (Applied Biosystems Division of Perkin-Elmer Corp., Foster City, Calif.). Oligonucleotides labeled with 6-FAM were synthesized using the manufacturer's suggested modifications to the synthesis cycle (Applied Biosystems Inc., 1994) and were subsequently deprotected at 55° C. for 4 hr. LDR oligonucleotides were purified by ethanol precipitation after overnight deprotection at 55° C. The primer-specific portions of the oligonucleotides used for PCR amplification were purified by polyacrylamide gel electrophoresis on 10% acryl amide/7M urea gels. Oligonucleotides were visualized after electrophoresis by UV shadowing against a lightening screen and excised from the gel (Applied Biosystems Inc., 1992). They were then eluted overnight at 64° C. in TNE (i.e. Tris-sodium EDTA) buffer (100 mM Tris/HCl pH 8.0 containing 500 mM NaCl and 5 mM EDTA) and recovered from the eluate using Sep Pak cartridges (Millipore Corp, Milford, Mass.) following the manufacture's instructions.

Oligonucleotides were resuspended in 100 μl TE (i.e. 10 mM Tri-HCl pH 8.0 containing 1 mM EDTA). Typical concentrations of these original LDR probe solutions are about 1 μg/μl or approximately 74 pm/μl as determined by the following formula:

$$[\text{concentration } (\mu g/\mu l)\times 10^6)]/[\text{length } (nt)\times 325]=pm/\mu l$$

The concentrations of the LDR probes are given in Table 1. The concentrations of oligonucleotides complementary to the oligonucleotide probes of the ligase detection reaction were higher. ZipALg1F was 3.75 μg/μl and ZipBLg2R was 2.01 μg/μl or 524 pm/μl and 281 pm/μl, respectively, as determined by the formula above.

TABLE 1

Primer	Length	µg/µl	pm/µl Vol = 100 pm	l = 200 pm
G6PDEx6-3L	48 nt	0.86	55.1 1.81 µl	3.63 µl
G6PDEx6-4R	48	0.65	41.7 2.4	4.8
ErbBEx1-5L	48	0.95	60.9 1.64	3.28
ErbBEx1-5R	48	1.4025	89.9 1.11	2.22
Int2Ex3-7L	50	1.6005	98.5 1.02	2.03
Int2Ex3-8R	46	1.306	87.4 1.14	2.29
p53Ex8-9L	2	1.036	61.3 1.63	3.26
p53Ex8-10R	44	1.164	81.4 1.23	2.46
SODEx3-11L	49	1.287	80.8 1.24	2.48
SODEx3-12R	47	1.2045	78.9 1.27	2.53

As a prerequisite for the LDR phase, the downstream LDR oligonucleotides probes were phosphorylated with T4 polynucleotide kinase. Aliquots of the 5 downstream oligonucleotides equivalent to 200 pm (see Table 1) were combined with 10 µl of 10× kinase buffer (500 mM Tris/HCl pH 8.0, 100 mM $MgCl_2$), 10 µl of 10 mM ATP, 20 U T4 kinase, and sufficient water-ME to give a final volume of 100 µl. Phosphorylation was carried out at 37° C. for 30 min followed by incubation for 10 min at 85° C. to inactivate the T4 enzyme. The resulting concentration of the kinased LDR probe solution was 2 pm/µl or 2000 fm/µl in each probe.

The kinase reaction is summarized as follows:

4.8 µl	G6PDEx6-4R
2.2 µl	ErbBEx1-5R
2.3 µl	Int2Ex3-8R
2.5 µl	p53Ex8-10R
2.5 µl	SODEx3-12R
10 µl	10 x Kinase Buffer
10 µl	10 mMATP
65.7 µl	HOH + ME
100 µl	Total
+ 2 µl	= 20 units T4 Kinase

37° C. for 30 min.
Heat kill kinase, 85° C., 10 min.
Final concentration = 2 pm/µl = 2000 fm/µl

The solutions of the LDR and PCR oligonucleotides were adjusted to convenient concentrations. The kinased LDR probe solution was diluted fourfold in water to yield a concentration of 500 fm/µl. A solution of the upstream LDR probes was made by combining volumes of the probes equivalent to 200 pm (see-Table 1) with sufficient water to give a final volume of 400 µl. This created a solution 500 fm/µl in each of the upstream LDR probes. Aliquots (20 µl) of the kinased and unkinased LDR probes were frozen for subsequent use. Standard solutions of the PCR primers (10 pm/µl) were prepared from their original solutions by combining 9.5 p of ZipALg1F and 17.8 µl of ZipBLg2R with sufficient water to achieve a total volume of 500 µl. These solutions were frozen for use in the LDR/PCR process. Unkinased probes were prepared according to the following:

200 pm	ea Primer
3.62 µl	G6PDEx6-3L
3.28 µl	ErbBEx1-5L
2.04 µl	Int2Ex3-7L
3.26 µl	p53Ex8-9L
2.48 µl	SODEx3-11L
385.32 µl	HOH
400 µl	Total Vol

Final concentration = 0.5 pm/µl = 500 fm/µl

TABLE 2

		Sequences Probe (length in nt)		
Gene	Location	Upstream	Downstream	Ligation Position
erb	17q12–q21	erbBEx1-5L (48)	erbBEx1-6R (48)	exon "1" P40
G6PD	Xq28	G6PDEx6-3L (48)	G6PDEx6-4R (48)	exon 6 W1145
Int2	11q13	Int2Ex3-7L (50)	Int2Ex3-8R (46)	exon 3 W135
p53	17p13.1	p53Ex8-9L (52)	p53Ex8-10R (44)	exon 8 P51
SOD	21q22.1	SODEx3-11L (49)	SODEx3-12R (47)	exon 3 P355

FIG. **24** shows the design of LDR oligonucleotide probes for quantification of gene amplifications and deletions in the LDR/PCR process. These oligonucleotide probes were designed to recognize exon 8 in the p53 tumor suppressor gene (on chromosome 17p), exon 3 of int-2 (on chromosome 11 q), an internal exon in HER-2/neu (i.e. HER-2/neu/erbB oncogene) (on chromosome 17q), exon 3 in SOD. (i.e. super oxide dimutase) (on chromosome 21(q), and exon 6 in G6PD (i.e. glucose 6-phosphate dehydrogenase) (on chromosome Xq). Each pair of LDR oligonucleotide probes has the following features: (i) The left oligonucleotide probe contains from 5' to 3' an 18 base sequence identical to the fluorescently labeled secondary oligonucleotide primer (black bar), an "adjustment sequence" (white bar), and a target-specific sequence of from 22 to 28 bases with a T_m of 75° C. (patterned bar); (ii) The right oligonucleotide probe contains from 5' to 3' a target-specific sequence of 20-25 bases with a T_m of 75° C. (patterned bar), a single HaeIII or HinPII restriction site at slightly different positions within the target-specific sequence, and an "adjustment sequence" (white bars). The two oligonucleotide probes are designed such that their combined length is exactly 96 bases, with 50 G+C bases and 46 A+T bases. The position of each unique restriction site generates a product which differs by at least 2 bases from the other products. Each oligonucleotide probe set has an exon-specific region chosen to ligate the junction sequence of (A, T)C C(A, T). This junction sequence corresponds to either a proline residue (codon CCN) or the complementary sequence of a tryptophan residue (TGG). These sequences were chosen to minimize differences in ligation rates and the chance of a polymorphism at the ligation junction.

LDR Probe Sequences

G6PDEx6-3L
5'CAC GCT ATC CCG TTA GAC ATT GTC AAG CAG GCG ATG TTG TCC CGG TTC 3' (SEQ. ID. NO. 1)

G6PDEx6-4R
5'CAG ATG GGG CCG AAG ATC CTG TTA TTG ATA CAT AGT GCG GTA GTT GGC 3' (SEQ. ID. NO. 2)

erbBEx1-5L
5'CAC GCT ATC CCG TTA GAC ATC GCC CTG ATG GGG AGA ATG TGA AAA TTC 3' (SEQ. ID. NO. 3)

erbBEx1-6R
5'CAG TGG CCA TCA AAG TGT TGA GGG AGC GTA CAT AGT GCG GTA GTT GGC 3' (SEQ. ID. NO. 4)

Int2Ex3-7L
5'CACGCTATCCCGTTAGAC ATT CAT AAC CCT TGC CGT TCA CAG ACA CGT AC 3' (SEQ. ID. NO. 5)

Int2Ex3-8R
5'CAC AGT CTC TCG GCG CTG GGC AAT AAT ACA TAG TGC GGT AGT TGG C 3' (SEQ. ID. NO. 6)

p53Ex8-9L
5'CAC GCT ATC CCG TTA GAC ATC TTA GTA ATT GAG GTG CGT GTT TGT GCC TGT C 3' (SEQ. ID. NO. 7)

p53Ex8-10R
5'CTG GGA GAG ACC GGC GCA CAT TAC TAC ATA GTG CGG TAG TTG GC 3' (SEQ. ID. NO. 8)

SODEx-3-11L
5'CACGCTATCCCGTTAGAC ATC TGT ACC AGT GCA GGT CCT CAC TTT AAT C 3' (SEQ. ID. NO. 9)

(SEQ. ID. NO. 10)
SODEx-3-12R
5'CTC TAT CCA GAA AAC ACG GTG GGC CGC TACATAGTGCGGTAGTTGGC 3'

PCR Primers:

ZipALg1F
5' Fam-GGA GCA CGC TAT CCC GTT AGA C 3' (SEQ. ID. NO. 11) (Tm = 71° C.)

ZipBLg2R
5' CGC TGC CAA CTA CCG CAC TAT G 3' (SEQ. ID. NO. 12) (Tm = 72° C.)

(Underlined sequences are common between LDR probes and ZipALg1F or the complement of ZipBLg2R.)

Example 3

Buffers and Reagents

A. LDR Buffers/Reagents—the following LDR buffers and reagents were selected:

10×ST ligase buffer (0.2 M Tris pH 8.5, 0.1 M $MgCl_2$) [This was also tested with Tris at pH 7.6.]

10×TT ligase buffer (0.2 M Tris pH 7.6, 0.5 M KCl, 0.1 M MgCl2, 5 mM EDTA)

NAD (10 mM)

DTT (200 mM)

LDR primer solution containing one tenth concentration of each of the LDR primer mixtures (50 fin of each LDR primer per μl)

Tth DNA Ligase (625 U/μl)

PCR Buffers/Reagents—the following PCR buffers and reagents were selected:

10× Stoffel buffer (0.1 M KCl 0.1M Tris-HCl pH 8.3 Perkin Elmer)

dNTP solution (100 mM total, 25 mM of each dNTP Perkin Elmer), diluted 5 fold in dHOH to a final concentration of 5 mM of each dNTP

ZipALg1F (10 pm/μl)

ZipBLg2R (10 pm/μl)

Example 4

LDR/PCR Process

Four LDR/PCR processes were performed for each DNA to be tested with reaction tubes PCR amplified to 22, 24, 26, and 30 cycles to assure that one reaction would be halted in the exponential phase. Each LDR reaction (20 μl) was thermal cycled and then a PCR mix (30 μl) containing primers with a portion complementary to the primer-specific portion of the LDR probes was added to each specimen to allow exponential amplification. To minimize differences between reaction tubes, master mixes of LDR and PCR reagents were made.

A master mix of LDR reagents was constructed with a volume sufficient for all reaction tubes. Proportions and volumes for a single reaction were as follows:

Reagent	Volume
10X ST Ligase Buffer	2 μl
NAD (10 mM)	2 μl
DTT (200 mM)	1 μl
dHOH_	5 μl
Total	10 μl
Tth DNA Ligase	0.2 μl (=125 U)

Mixes of target DNA and LDR probes for each reaction tube were constructed with the following proportions:

Reagent	Volume
DNA (TaqI digested)	1 μl (=50 ng)
LDR Probe Mix	4 μl (200 fm each primer)
dHOH_	5 μl
Total	10 μl

For each reaction, 10 μl was placed in a thin-walled PCR tube, rapidly mixed with 10 μl of LDR reagent mix (including ligase), overlayed with mineral oil, and placed in a Perkin Elmer 9600 thermal cycler.

LDR was initiated by holding at 96° C. for 2 minutes to denature the DNA followed by 10 cycles of 94° C. for 30 seconds and 65° C. for 4 minutes.

PCR reagent mixes for each reaction tube were constructed with the following proportions:

Reagent	Volume
10X Stoffel buffer	5 μl
dNTP solution	8 μl (=0.8 mM each dNTP in final reaction)
ZipALg1F (10 pm/μl)	2.5 μl (=25 pm per reaction)
ZipBLg2R (10 pm/μl)	2.5 μl (=25 pm per reaction)
dHOH	12 μl
Total	30 μl
Stoffel Fragment	0.25 μl (=2.5 U)

At the completion of the LDR reaction, the tubes were held at 94° C., while 30 ml of PCR reagent mix (including Stoffel fragment) were added to each tube. PCR amplification was accomplished by thermal cycling at 94° C. for 15 seconds followed by 60° C. for 50 seconds. At 22, 24, 26, and 30 cycles, respectively, one of four identical reaction tubes of each DNA specimen was removed and quenched in a slurry of dry ice and ETOH.

Example 5

Agarose Gel Evaluation

Ten microliter aliquots of the 26 and 30 cycle reaction specimens were evaluated on a 2% agarose gel. Ethidium bromide staining revealed bands of the expected size (104 bp).

Example 6

Digestion of Products, Preparation of Dilutions, and Loading on GeneScanner

To separate the gene-specific LDR/PCR products, 10 μl aliquots of the 22, 24, and 26 cycle reactions were digested by adding 10 μl of a solution containing 5 U each of HaeIII and HinPlI restriction enzymes (both from New England BioLabs), 2 μl of 10× restriction enzyme buffer number 2 (New England BioLabs), and 8 μl of dHOH (i.e. distilled water). The digests were incubated at 37° C. for one hour and then stopped by the addition of 1 μl of 0.5 M EDTA, pH 8.0. The restriction digests were a one half dilution of the original LDR/PCR products. A 10 fold dilution of each sample was also prepared by adding 5 μl of each restriction digest to 20 μl of TE buffer.

Before loading samples on the ABI 373A DNA-Sequencer (Applied Biosystems) a 1:5 mixture of 50 mM EDTA pH 8.0 containing 2% blue dextran and de-ionized formamide was made. To 5 μl of the EDTA-Blue Dextran solution, 5 μl of digested LDR/PCR product dilution and 1 μl of GENESCAN 1000 ROX marker (Applied Biosystems) were added. These solutions were heated to 85° C. for 10 minutes and snap chilled on ice, before 5.5 μl were loaded on the denaturing gel.

Samples were analyzed in an Applied Biosystems 373A DNA sequencer on a 0.4 mm thick, 10% polyacrylamide/7M urea gel with a well-to-read distance of 12 cm. The gel matrix was buffered with 1.2×TBE (106 mM Tris-borate and 2.4 mM EDTA pH 8.3) and the electrophoresis chamber buffer contained 0.6×TBE (53.4 mM Tris-borate and 1.2 mM EDTA pH 8.3). The gel was pre-run prior to sample loading at 1600 V for 30 minutes with the electrode polarity reversed (anode in the chamber with sample wells at the top of the gel). After loading, the gene-specific LDR/PCR products were electrophoresed at 1200 V and the primary data was captured using the ABI 672 Data Collection Software V1.1 (Applied Biosystems)

Using the ABI 672 GeneScan Analysis Software V1.2.2 (Applied Biosystems), the resulting data were displayed as electropherograms, with peak heights and peak areas calculated.

In the normal female, the ErbB2 peak is lower, and the p53 peak is slightly lower than the remaining 3 peaks. See FIGS. **25**A-D. In different experiments, it was observed that the ErbB2 peak is always lower, the G6PD, Int-2, p53, and SOD peak areas would vary somewhat, but all 5 peaks would retain the same relative profile from one sample to the next for a given experiment. When comparing male with female DNA, the G6PD peak was about half the area of other peaks, consistent with a single X-chromosome in males, while the other peaks were essentially the same. The ErB2 peak for the NM10 Breast Cancer cell line is slightly elevated, while that in cell line SKBR3 is several fold-greater than the normal female control, reflecting the known ErbB-2 gene amplification in these two cell lines. In addition, cell line NM10 appears to have undergone LOH (i.e. a loss of heterozygosity) of p53, while cell line SKBR3 appears to have undergone LOH of G6PD and p53. Some of the cells in cell line SKBR3 may have lost both copies of the p53 gene. Repeating these amplifications in the absence of the ErbB-2 primers was used to confirm the presence of these additional gene deletions (see below).

These results can be quantified by comparing the ratio of peak areas in each peak to a standard (the SOD peak area) for that experiment. The raw data and ratio of peak areas are given below:

TABLE 3

Raw Peak Area Data

	Genes				
	ErB	G6PD	Int2	p53	SOD
Male	9954	21525	45688	36346	62506
Female	8340	39309	39344	30270	54665
NM10	20096	55483	67083	17364	84339
SKBR3	106650	19120	50103	2888	48119

TABLE 4

Ratio of Peak Areas to SOD Peak Area

	ErbB/SOD	G6PD/SOD	Int2/SOD	p53/SOD
Male	0.16	0.34	0.73	0.58
Female	0.15	0.72	0.72	0.55
NM10	0.24	0.66	0.80	0.21
SKBR3	2.22	0.40	1.04	0.06

Although the ratios differ for each gene, (due to different efficiencies of LDR/PCR for each gene) the ratios are generally consistent between the male and female sample, except for the G6PD/SOD ratio. The G6PD for the female is about twice the value as the male, accurately reflecting the presence of two and one X chromosome, respectively. One can quantify the amount of ErbB2 amplification by comparing the ratio of peak area ratios between normal DNA and cancer cell lines.

TABLE 5

Ratio of Peak Areas Ratios

	ErbB/2	G6PD	Int2	p53
Female/Male	0.96	2.09	0.98	0.95
NM10/Male	1.50	1.91	1.09	0.35
SKBR3/Male	13.92	1.15	1.42	0.10

From these ratios, it can be determined that the normal male and female have the same number of genes on chromosomes 17q (ErbB), 17p (p53), and 11 q (Int 2), but that the female has twice as many G6PD genes, or X chromosomes. Likewise, cell line NM10 showed slight amplification of the ErbB-2 gene, and LOH at p53, while cell line SKBR3 shows significant amplification of the ErbB-2 gene, LOH at G6PD and p53. To confirm additional gene amplifications and deletions, primer pairs causing massive amplifications may be removed from the LDR/PCR reaction (see below).

In the normal female, the ErbB2 peak is lower than the remaining 4 peaks. In different experiments, it was observed that the G6PD, Int-2, p53, and SOD peak areas would vary somewhat, but would retain the same relative profile from one sample to the next. See FIGS. **26**A-C. The ErbB2 peak was consistently lower, and slight shoulders were observed on the G6PD and SOD peaks, for unknown reasons. The ErbB-2 peak in both cell line samples is several fold greater than the normal female control, reflecting the known ErbB-2 gene amplification in these two cell lines. In addition, the ZR-75-30 line appears to show LOH of p53, while the SKGT-2 cell line appears to have a slight amplification of the Int-2 region. By repeating these LDR/PCR experiments in the absence of the ErbB-2 primers, it was demonstrated that these results are not artifacts of the massive levels of ErbB-2 amplification. See FIGS. **27**A-C. Both gene amplifications and deletions for multiple genes using the LDR/PCR format have been demonstrated. See FIGS. **26**A-C and 27A-C.

Again, these results can be quantified by comparing the ratio of peak areas in each peak to a standard (the SOD peak area) for that experiment. The raw data and ratio of peak areas are given below:

TABLE 6

Raw Peak Area Data

	Genes				
	ErbB	G6PD	Int2	p53	SOD
Female; 4 Primer Sets	NA	9577	8581	9139	8128
ZR7530; 4 Primer Sets	NA	8452	7904	4168	7996
SKGT2; 4 Primer Sets	NA	15915	28614	13116	12478
Female; 5 Primer Sets	3955	9436	8066	9304	8848
ZR7530; 5 Primer Sets	66748	11105	8812	4163	9303
SKGT2; 5 Primer Sets	263254	21877	31887	13630	13480

TABLE 7

Ratio of Peak Areas to SOD Peak Area

	ErbB/SOD	G6PD/SOD	Int2/SOD	p53/SOD
Female; 4 Primer Sets	NA	1.18	1.06	1.12
ZR7530; 4 Primer Sets	NA	1.06	0.99	0.52
SKGT2; 4 Primer Sets	NA	1.28	2.29	1.05

TABLE 7-continued

Ratio of Peak Areas to SOD Peak Area

	ErbB/SOD	G6PD/SOD	Int2/SOD	p53/SOD
Female; 5 Primer Sets	0.45	1.97	0.91	1.05
ZR7530; 5 Primer Sets	7.17	1.19	0.95	0.45
SKGT2; 5 Primer Sets	19.53	1.62	2.37	1.01

The ratios are remarkably consistent between the four primer set and the five primer set experiments. The only exception is the G6PD peak for the SKGT2 cell line, where the huge peak for ErbB-2 may have added to the G6PD peak.

One can quantify the amount of ErbB2 and Int-2 amplification as well as p53 deletion by comparing the ratio of peak area ratios between normal DNA and cancer cell lines, as shown in Table 8. In addition, the ratios from using 4 sets of primers can be compared with 5 sets of primers to ascertain the internal consistency of this technique.

TABLE 8

Ratio of Peak Area Ratios

	ErbB	G6PD	Int2	p53
Female; 4/5	NA	1.10	1.16	1.07
ZR7530; 4/5	NA	0.89	1.04	1.16
SKGT2; 4/5	NA	0.79	0.97	1.04
ZR7530/Female; 4/4	NA	0.90	0.94	0.46
ZR7530; Female; 5/5	16.05	1.12	1.04	0.43
SKGT2/Female; 4/4	NA	1.08	2.17	0.93
SKGT2; Female; 5/5	43.69	1.52	2.59	0.96

The values for the top half of Table 8 should all be close to 1.0 if the LDR/PCR technique is internally consistent, when using 4 or 5 primers. All values are very close to 1.0. Again, the value for G6PD for SKGT2 is a bit low for the reasons mentioned.

The values on the bottom half of Table 8 show the extent of ErbB-2 amplification. The numbers are quite consistent for the 4 primer and 5 primer amplifications (with the exception of SKGT2-G6PD noted above). The ZR7530 cell line demonstrates a clear LOH for p53, while the SKGT2 cell line shows amplification of the Int-2 region, and both p53 genes present.

Primary PCR/Secondary PCR/LDR Process

Example 7

Oligonucleotide Synthesis

Oligonucleotides were assembled by standard phosphoramidite chemistry on an Expedite DNA synthesizer (Perseptive Biosystems, Framingham, Mass.). Oligonucleotides 5'-end labeled with 6-FAM, TET, and HEX were synthesized using the appropriate dye phosphoramidites (Perkin Elmer-Applied Biosystems) and purified with Oligonucleotide Purification Cartridges (Perkin Elmer-Applied Biosystems) following the manufacturer's protocol (Applied Biosystems Division-Perkin Elmer Corp., "Synthesis and Purification of Fluorescently Labeled Oligonucleotides Using Dye Phosphoramidites," *User Bulletin*, number 78, Applied Biosystems Division, Foster City, Calif., (1994)), which is hereby incorporated by reference. All oligonucleotides were checked for purity on an Applied Biosystems 270-HT capillary electrophoresis instrument using a mPAGE-3 column

(J&W Scientific, Folsom, Calif.). Only oligonucleotides that were greater than 95% pure were used for the experiments. Oligonucleotides were resuspended in 250 ml TE (10 mM Tris/HCl and 5 mM EDTA pH 8.0). Typical concentrations were 300-500 mM for crude stock solutions and 100-200 mM for OPC (i.e. Oligonucleotide Purification Columns available from Applied Biosystems) purified stock solutions. For PCR and LDR, oligonucleotides were diluted to working solutions of 10 mM (10 pmoles/ml) or 5 mM (5 pmoles/ml).

Example 8

Phosphorylation of LDR Oligonucleotides

The 12 LDR common oligonucleotides were phosphorylated at the 5' end to permit ligation to the fluorescent labeled oligonucleotides. The oligonucleotides are shown below in Table 9.

TABLE 9

LDR Oligonucleotide Sequences

Locus	Allele-Specific Oligonucleotide (5' -> 3')	Common Oligonucleotide (5' -> 3')
1	FAM-AGCTTCAATGATGAGAACCTGC (SEQ. ID. NO. 13)	P-GCATAGTGGTGGCTGACCTGTTCATAT (SEQ. ID. NO. 14)
	TET-AGCTTCAATGATGAGAACCTGT (SEQ. ID. NO. 15)	
2	FAM-CTCCATGGGCCCAGCC (SEQ. ID. NO. 16)	P-AGCACTGGTGCCCTGTGAG (SEQ. ID. NO. 17)
	TET-CTCCATGGGCCCAGCT (SEQ. ID. NO. 18)	
3	FAM-GGGGACAGCCATGCACTGA (SEQ. ID. NO. 19)	P-GCCTCTGGTAGCCTTTCAACCATA (SEQ. ID. NO. 20)
	TET-GGGGACAGCCATGCACTGC (SEQ. ID. NO. 21)	
4	FAMTTAGAAATCATCAAGCCTAGGTCAT (SEQ. ID. NO. 22)	P-CACCTTTTAGCTTCCTGAGCAATGAT (SEQ. ID. NO. 23)
	TET-TTAGAAATCATCAAGCCTAGGTCAG (SEQ. ID. NO. 24)	
5	HEX-GGTTGTATTTGTCACCATATTAATTA (SEQ. ID. NO. 25)	P-ATTTTTCTCTATTGTTTTCATCTTTCAGGA (SEQ. ID. NO. 26)
	HEX-ATGGTTGTATTTGTCACCATATTAATTG (SEQ. ID. NO. 27)	
6	FAM-GGGCCAAGAAGGTATCTACCA (SEQ. ID. NO. 28)	P-ATAGTGTCTATTAGGCATTTGAAAATGTGTAT (SEQ. ID. NO. 29)
	TET-GGGCCAAGAAGGTATCTACCG (SEQ. ID. NO. 30)	
7	FAM-ACACAGCAGCTTACTCCAGAGG (SEQ. ID. NO. 31)	P-TCAAGTCCAAGGCCATTGGCTTATA (SEQ. ID. NO. 32)
	TET-ACACAGCAGCTTACTCCAGAGA (SEQ. ID. NO. 33)	
8	FAM-CCAGCAAAGAGAAAAGAAGGG (SEQ. ID. NO. 34)	P-CCCCAGAAATCACAGGTGGGCTAT (SEQ. ID. NO. 35)
	TET-CCAGCAAAGAGAAAAGAAGGA (SEQ. ID. NO. 36)	
9	FAM-ATGATATTAGAGCTCACTCATGTCCA (SEQ. ID. NO. 37)	P-TCAGTTTGGAAAAAGACAAAGAATTCTTT (SEQ. ID. NO. 38)
	TET-ATGATATTAGAGCTCACTCATGTCCG (SEQ. ID. NO. 39)	
10	HEX-TGCTGTCTTCCAGGAATCTGTT (SEQ. ID. NO. 40)	P-CAACTCTCTCGAAGCCATGTTCACAA (SEQ. ID. NO. 41)
	HEX-ATTGCTGTCTTCCAGGAATCTGTG (SEQ. ID. NO. 42)	

TABLE 9-continued

LDR Oligonucleotide Sequences

Locus	Allele-Specific Oligonucleotide (5' -> 3')	Common Oligonucleotide (5' -> 3')
11	HEX-GGACATAGTGACCGTGCAGGTC (SEQ. ID. NO. 43)	P-CTTCCCCAGTGTGAGTGCCGTA (SEQ. ID. NO. 44)
	HEX-ATGGACATAGTGACCGTGCAGGTT (SEQ. ID. NO. 45)	
12	HEX-CTATGACACCGTCATCAGCAGG (SEQ. ID. NO. 46)	P-GACATCCAGGCCCCCGAC (SEQ. ID. NO. 47)
	HEX-TACTATGACACCGTCATCAGCAGA (SEQ. ID. NO. 48)	

The allele-specific oligonucleotides are 5' end labeled with either FAM, TET, or HEX. All the common oligonucleotides are phosphorylated at the 5' end. Underline denotes tails that are not complementary to the target sequence. LDR primer sets were designed in two ways: (i) allele-specific primers were of the same length but contained either FAM or TET label; or (ii) the allele-specific primers were both labeled with HEX but differed in length by two bases.

This was accomplished either during the synthesis with Phosphate-ON (Clontech Laboratories, Palo Alto, Calif.) according to the manufacturer's instructions or post-synthesis, using T4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, Ind.). In the latter, a common oligomer was diluted into 50 μl of kinase buffer (50 mM Tris/HCl, 10 mM $MgCl_2$, and 1 mM ATP) to a final concentration of 1 mM (500 pmol in 50 μl). Ten units of T4 kinase was added, and the reaction was incubated at 37° C. for 30 min. The T4 kinase was inactivated by heating at 95° C. for 10 min.

The kinase reaction was carried out as follows:

10 μl	50 mM common oligo
5 μl	10x kinase buffer
5 μl	10 mM ATP
30 μl	H20
50 μl	Total
+ 1 μl	T4 Kinase (10 units)

37° C. for 30 min.
95° C. for 10 min.
Final concentration = 10 mM

Example 9

Multiplex PCR Amplification

Twelve gene regions (2-13) were chosen for simultaneous PCR amplification based on information available in the Human Genome Database, as shown below in Table 10.

TABLE 10

List of Polymorphic Sites Analyzed

Site Number	Locus Symbol	Locus Name	Chr. Location	Nucleotide Position	Variation	Het.	Ref.
1	CYP2D6	cytochrome P450IID6	22q13.1	4469 (M33388)	C, T	.38	2
2	AT3	antithrombin III	1q23–q25.1	7987 (X68793)	C, T	.46	3
3	C6	complement component C6	5p14–p12	185 (X72179)	A, C	.47	4
4	IL1A	interleukin 1 alpha	2q13	6282 (X03833)	T, G	.34	5
5	NF1	neurofibromatosis	17q11.2	63683 (L05367)	A, G	.47	6
6	ALDOB	aldolase B	9q22.3–q31	1087 (M15656)	A, G	.50	7
7	A2M	alpha 2 macroglobulin	12p13.3–p12.3	153 (X68731)	G, A	.42	8
8	IGF2	insulin growth factor	11p15.5	820 (X07868)	G, A	.46	9
9	PROS1	protein S alpha	3p11–cen	183 (M36564)	A, G	.50	10
10	LIPC	triglyceride lipase	15q21–q23	113 (M29189)	T, G	.49	11

TABLE 10-continued

List of Polymorphic Sites Analyzed

Site Number	Locus Symbol	Locus Name	Chr. Location	Nucleotide Position	Variation	Het.	Ref.
11	CD18	integrin B-2 subunit	21q22.3	109 (X64081)	C, T	.50	12
12	LDLR	low density lipoprotein receptor	19p13.2	70 (L00344)	G, A	.50	13

The site numbers are specific single point variations located within the respective genes. All variations indicated are defined on the sense strand. Genbank accession numbers are indicated in parentheses. Chr., chromosome; Het., heterozygosity.

References in Table 10

2 M. Armstrong, et al., "A Polymorphic Cfo I Site In Exon 6 of the Human Cytochrome P450 CYPD6 Gene Detected by the Polymerase Chain Reaction," Human Genetics 91: 616–17 (1993), which is hereby incorporated by reference.

3 S. C. Bock, et al., "Antithrombin III Utah: Proline-407 to Leucine Mutation in a Highly Conserved Region Near the Inhibitor Reactive Site," Biochemistry 28: 3628 (1991), which is hereby incorporated by reference.

4 G. Dewald, et al., "Polymorphism of Human Complement Component C6: An Amino Acid Substitution (glu/ala) Within the Second Thrombospondin Repeat Differentiates Between the Two Common Allotypes C6 A and C6 B," Biochem. Biophys. Res. Commun. 194: 458–64 (1993), which is hereby incorporated by reference.

5 P. A. Velden, et al., "Amino Acid Dimorphism in IL1A is Detectable by PCR Amplification," Hum. Mol. Genet. 2: 1753 (1993), which is hereby incorporated by reference.

6 R. M. Cawthon, et al., "Identification and Characterization of Transcripts From Theneurofibromatosis 1 Region: The Sequence and Genomic Structure of EV12 and Mapping of Other Transcripts," Genomics 7: 555–65 (1990), which is hereby incorporated by reference.

7 C. C. Brooks, et al., "Association of the Widespread A149P Hereditary Fructose Intolerance Mutation With Newly Identified Sequence Polymorphisms in the Aldolase B Gene," Am. J. Human Genetics 52: 835–40(1993), which is hereby incorporated by reference.

8 W. Poller, et al., "Sequence Polymorphism in the Human Alpha-2-Macroglobulin (A2M) Gene," Nucleic Acids Res. 19: 198 (1991), which is hereby incorporated by reference.

9 T. Gloudemans, "An Ava II Restriction Fragment Length Polymorphism in the Insulin-Like Growth Factor II Gene and the Occurrence of Smooth Muscle Tumors," Cancer Res. 53: 5754–58 (1993), which is hereby incorporated by reference.

10 C. M. Diepstraten, et al., "A CCA/CCG Neutral Dimorphism in the Codon for Pro 626 of the Human Protein S Gene PSa (PROS1)," Nucleic Acids Res. 19: 5091 (1991), which is hereby incorporated by reference.

11 M. Reina, et al., "SSCP Polymorphism in the Human Hepatic Triglyceride Lipase (LIPC) Gene," Hum. Mol. Genet. 1: 453 (1992), which is hereby incorporated by reference.

12 S. Mastuura, et al., "Investigation of the Polymorphic AvaII Site by a PCR-based Assay at the Human CD18 Gene Locus," Human Genetics 93: 721 (1994), which is hereby incorporated by reference.

13 L. Warnich, et al., "Detection of a Frequent Polymorphism in Exon 10 of the Low-Density Lipoprotein Receptor Gene," Human Genetics 89: 362 (1992), which is hereby incorporated by reference.

Each region was well characterized and harbored a single-base variation with only two known alleles. PCR amplifications were performed using genomic DNA isolated from whole blood using the Purgene DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, Mich.) according to the manufacturer's instructions.

A volume of 25 μl of PCR buffer (10 mM Tris/HCl pH 8.3, 10 nM KCl, 4 mM $MgCl_2$, 0.4 mM each dNTP), 10-100 ng of genomic target DNA, PCR hybrid primer pairs 1-12 (2 pmol of each primer), and 1.3 units of AmpliTaq DNA polymerase Stoffel fragment (Applied Biosystems) was placed in a thin-walled MicroAmp reaction tube (Applied Biosystems). Each hybrid primer consisted of a gene-specific 3' region (16-29 bases) and a 5' region (22 bases) corresponding to one of two sets of universal (i.e. the portions which are primer-specific) primers (See FIG. **4**, where F1=Tet, F2=Fam, and F3=Het). These primers are shown in Table 11.

TABLE 11

Primary PCR Primer Sequences

Number or Name	Primer (5' -> 3')	
1	F GGAGCACGCTATCCCGTTAGACAGCCAAGGGGAACCCTGAGAG	(SEQ. ID. NO. 49)
	R CGCTGCCAACTACCGCACTATGATCGTGGTCGAGGTGGTCACCATC	(SEQ. ID. NO. 50)
2	F CCTCGTTGCGAGGCGTATTCTGTATTTCCTCTTCTGTAAAAGGGAAGTTTGT	(SEQ. ID. NO. 51)
	R GCGACCTGACTTGCCGAAGAACATGTCCCATCTCCTCTACCTGATAC	(SEQ. ID. NO. 52)
3	F GGAGCACGCTATCCCGTTAGACTAAAGATCTGTCTTGCGTCCCAGTCA	(SEQ. ID. NO. 53)
	R CGCTGCCAACTACCGCACTATGTATCAATTTTGCAGAGCTTAGATGGAATG	(SEQ. ID. NO. 54)

TABLE 11-continued

Primary PCR Primer Sequences

Number or Name	Primer (5' -> 3')	
4	F CCTCGTTGCGAGGCGTATTCTGTAGCACTTGTGATCATGGTTTTAGAAATC	(SEQ. ID. NO. 1)
	R GCGACCTGACTTGCCGAAGAACTATCGTATTTGATGATCCTCATAAAGTTG	(SEQ. ID. NO. 56)
5	F GGAGCACGCTATCCCGTTAGACATCAGCCACTTGGAAGGAGCAAAC	(SEQ. ID. NO. 57)
	R CGCTGCCAACTACCGCACTATGATGGACCATGGCTGAGTCTCCTTTAG	(SEQ. ID. NO. 58)
6	F CCTCGTTGCGAGCCGTATTCTGAACCAACACGGAGAAGCATTGTTTTC	(SEQ. ID. NO. 59)
	R GCGACCTGACTTGCCGAAGAACTATTAGCCTCAATCCTCATACTGACCTCTAC	(SEQ. ID. NO. 60)
7	FGGAGCACGCTATCCCGTTAGACATCTCCTAACATCTATGTACTGGATTATCTAAATG	(SEQ. ID. NO. 61)
	R CGCTGCCAACTACCGCACTATGATCTTACTCAAGTAATCACTCACCAGTGTTG	(SEQ. ID. NO. 62)
8	F CCTCGTTGCGAGGCGTATTCTGAATGAGTCAAATTGGCCTGGACTTG	(SEQ. ID. NO. 63)
	R GCGACCTGACTTGCCGAAGAACTTAATTCCCGTGAGAAGGGAGATG	(SEQ. ID. NO. 64)
9	F CCTCGTTGCGAGGCGTATTCTGAAGGATCTGGATGAAGCCATTTCTAAAC	(SEQ. ID. NO. 65)
	R GCGACCTGACTTGCCGAAGAACTTGGAAAAGGTATTATAAGCAGAGAAAAGATG	(SEQ. ID. NO. 66)
10	F GGAGCACGCTATCCCGTTAGACAGGACCGCAAAAGGCTTTCATC	(SEQ. ID. NO. 67)
	R CGCTGCCAACTACCGCACTATGTAGCACCCAGGCTGTACCCAATTAG	(SEQ. ID. NO. 68)
11	F CCTCGTTGCGAGCCGTATTCTGATCGGGCGCTGGGCTTCAC	(SEQ. ID. NO. 69)
	R GCGACCTGACTTGCCGAAGAACATCAGATGCCGCACTCCAAGAAG	(SEQ. ID. NO. 70)
12	F GGAGCACGCTATCCCGTTAGACATAAGAGCCCACGGCGTCTCTTC	(SEQ. ID. NO. 1)
	R CGCTGCCAACTACCGCACTATGTAAGAGACAGTGCCCAGGACAGAGTC	(SEQ. ID. NO. 72)
ZipALg1	FGGAGCACGCTATCCCGTTAGAC	(SEQ. ID. NO. 73)
ZipBLg2	RCGCTGCCAACTACCGCACATG	(SEQ. ID. NO. 74)
ZipCLg3	F CCTCGTGCGAGGCGTATTCTG	(SEQ. ID. NO. 75)
ZipDLg4	R GCGACCTGACTTGCCGAAGAAC	(SEQ. ID. NO. 76)

Solid underline denotes the ZipALg1 and ZipBLg2 sequences. Dotted underline denotes the ZipCLg1 and ZipDLg2 sequences. Linker sequences are indicated in bold. F = forward, R = reverse.

Forward and reverse hybrid primers for loci 1, 3, 5, 7, 10, and 12 contained 5' end regions identical to universal primers ALg1 and BLg2 respectively. Forward and reverse primers to loci 2, 4, 6, 8, 9, and 11 contained 5' end regions identical to universal primers CLg3 and DLg4, respectively. The rationale for using a low concentration of the hybrid primers in the PCR phase was to deplete the hybrid primers during the reaction. This would theoretically allow products with low amplification efficiencies to "catch up with" those that had high amplification efficiencies. Amplification was attained by thermal cycling for 1 cycle of 96° C. for 15 sec to denature, then 15 cycles of 94° C. for 15 sec to denature and 65° C. for 60 sec to anneal and extend.

An equal volume of PCR buffer containing a high concentration of the two pairs of universal primers (25 pmol of each primer; Table 2) and 1.3 units of Amplitaq DNA polymerase Stoffel fragment was added to the Microamp reaction tube, and thermal cycling was performed for another 25 cycles with the annealing temperature lowered to 55° C. The upstream universal primers ALg1 and CLg3 were fluorescently labeled with 6-FAM and TET, respectively. All thermal cycling was achieved with a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems).

The primary PCR process was carried out under the following conditions:

5.9 µl	H_2O
5 µl	primer pairs 1–12 (2 pmol each primer)
2.5 µl	10X Stoffel fragment
4 µl	25 mM $MgCl_2$
5 µl	2 mM dNTP stock (each)
2.5 µl	genomic DNA (10 ng)
0.13 µl	Stoffel Frag. (1.3 units)
25 µl	Total

The primary PCR cycling conditions were as follows:

96° C. 15"

94° C. 15", 65° C. 1'×15

65° C. Hold

The secondary PCR process was carried out under the following conditions:

13.37 µl	H_2O
5 µl	zip primer pairs (25 pmol each primer)
2.5 µl	10X Stoffel fragment
4 µl	25 mM $MgCl_2$
0.13 µl	Stoffel Frag. (1.3 units)
25 µl	Total

The secondary PCR cycling conditions were as follows:

94° C. 15", 55° C. 1'×25

4° C. Hold

The PCR products were separated on an Applied Biosystems 373 DNA sequencer. A 3 ml aliquot of PCR sample was mixed with 3 ml of formamide containing fluorescently labeled Genescan-2500 [TAMRA] size standard (Applied Biosystems). The formamide/standard solution was prepared by adding 50 ml Genescan-2500 size standard [TAMRA] to 450 ml of formamide. The sample was heated at 95° C. for 2 min, quick cooled in ice, and electrophoresed through a denaturing 8% polyacrylamide gel in an Applied Biosystems 373 DNA sequencer running Genescan version 1.2 software. The sizes of the fluorescently labeled products were automatically computed by the Genescan analysis software using the local Southern method. The electropherograms clearly showed 12 distinct products (FIG. **28**). The uniform amount of each product was attributed to the similar size of each amplicon and the use of the primary primers with portions complementary to the secondary primers, which annealed with identical affinities to the 12 amplicons without the need to carefully adjust reaction conditions. The computed sizes of the products, which ranged from 135 to 175 bp, matched exactly to their actual sizes (see Table 12).

TABLE 12

List of PCR and LDR Products

Site	PCR Products		LDR Product			
Number	Label	Size (bp)	Label	Size (bp)	Variation	Allele
1	6-FAM	143	6-FAM	49	C	A
			TET		T	B
2	TET	145	6-FAM	35	C	A
			TET		T	B
3	6-FAM	147	6-FAM	43	A	A
			TET		C	B
4	TET	149	6-FAM	51	T	A
			TET		G	B
5	6-FAM	155	HEX	56	A	A
				58	G	B
6	TET	157	6-FAM	53	A	A
			TET		G	B
7	6-FAM	159	6-FAM	47	G	A
			TET		A	B
8	TET	161	6-FAM	45	G	A
			TET		A	B
9	TET	165	6-FAM	55	A	A
			TET		G	B
10	6-FAM	167	HEX	48	T	A
				50	G	B
11	TET	173	HEX	44	C	A
				46	T	B

TABLE 12-continued

List of PCR and LDR Products

Site	PCR Products		LDR Product			
Number	Label	Size (bp)	Label	Size (bp)	Variation	Allele
12	6-FAM	175	HEX	40	G	A
				42	A	B

The dual labeling approach (Table 12) made it much easier to distinguish the products on the electropherograms (FIG. **28**, compare panel A with panels B and C).

Example 10

Multiplex Ligase Detection Reaction

To avoid any possibility of labeled PCR product interfering with the detection of ligation product sequences, PCR product amplified using unlabeled PCR universal primers served as the target for the LDR. The polymerase in the PCR was inactivated by either freeze-thawing or adding EDTA/proteinase K to a final concentration of 5 mM and 100 mg/ml, respectively, and heating to 37° C. for 30 min and 95° C. for 10 min.

Proteinase K digestion was carried out under the following conditions:

20 µl	PCR product
+ 2.5 µl	500 mg/ml proteinase K
25 µl	Total

60° C. for 60 min.
95° C. for 10 min to heat kill proteinase K.

Four microliters of PCR product was diluted in 20 µl of LDR mix containing 50 mM Tris/HCl pH 8.5, 50 mM KCl, 10 mM $MgCl_2$, 1 mM NAD+, 10 mM DTT, LDR oligonucleotide sets 1-12 (200 fmol of each oligonucleotide), and 10 units of *Thermus aquaticus* DNA ligase (Barany, F. and Gelfand, D., "Cloning, Overexpression, and Nucleotide Sequence of a Thermostable DNA Ligase-Encoding Gene," *Gene*, 109:1-11 (1991), which is hereby incorporated by reference). Each LDR oligonucleotide probe set consisted of two allele-specific oligonucleotides and a common oligonucleotide. Each pair of discriminating allele-specific oligonucleotide probes in the LDR oligonucleotide probe sets 1, 2, 3, 4, 6, 7, 8, and 9 were the same size, with one oligonucleotide labeled with 6-FAM and the other labeled with TET. For LDR oligonucleotide probe sets 5, 10, 11, and 12, each pair of allele-specific oligonucleotides differed by 2 bases (the larger oligonucleotide had a 5' tail that was not complementary to the target sequence), and both oligonucleotides were labeled with HEX. Thermal cycling was performed for 1 cycle of 95° C. for 2 min to denature, then 20 cycles of 95° C. for 30 sec to denature, and 65° C. for 4 min to ligate.

The LDR process was carried out as follows:

4 µl	PCR product
2 µl	LDR oligo sets 1–12 (200 fmol each oligo)
2 µl	10X T. ligase buffer
2 µl	10 mM NAD+
1 µl	200 mM DTT
9 µl	H_2O
20 µl	Total
+ 1 µl	Taq Ligase (10 units)

Stock of Ligase: 0.5 µl of 625 u/ml + 3.2 µl 10× buffer + 27.5 µl H_2O

LDR cycling conditions were as follows:

94° C. 2'

94° C. 30", 65° C. 4'×20

4° C. Hold

A 3 µl aliquot of LDR sample was mixed with 3 µl of formamide containing fluorescently labeled Genescan-2500 [TAMRA] size standard (Applied. Biosystems). The sample was heated at 95° C. for 2 min, quick cooled in ice, and electrophoresed through a denaturing 10% polyacrylamide gel in an Applied Biosystems 373 DNA sequencer running Genescan version 1.2 software. The sizes of the fluorescently labeled products were automatically computed by the Genescan analysis software using the local Southern method. LDR profiles of two individuals are shown in FIG. **29**. When each fluorescent dye was analyzed independently (FIG. **29**, panels B-D and F-H), it was very easy to determine the alleles present for each locus. A simple "A" or "B" code was assigned to each LDR product (Table 12) and used to score the genotypes. See Table 13 as follows:

TABLE 13

Genotypes Determined by PCR-LDR for 5 Individuals

	Individual Site Number											
	1	2	3	4	5	6	7	8	9	10	11	12
1	AB	AB	AB	AB	AB	AB	AB	AA	AA	AB	AB	AB
2	AA	BB	AB	BB	AB	AB	BB	AA	AB	AA	AA	AA
3	AB	AB	AB	BB	AA	BB	BB	AA	AA	AB	AB	AA
4	AA	AB	AA	AB	AB	AA	AB	AA	AB	BB	AB	AA
5	AA	AA	AA	AB	AB	AB	AB	AB	AA	AB	AB	BB

The first individual (FIG. **29**A-D; Table 13, individual 1) was heterozygous at polymorphic sites 1-7, and 10-12. Heterozygosity at sites 1-4 and 6-7 was indicated by the detection of both 6-FAM and TET labeled products (FIG. **29**, panels B and C) at the respective positions on the electropherograms. Heterozygosity at sites 5 and 11-12 was indicated by the presence of two HEX labeled products, differing in size by 2 bases, for each of these loci (FIG. **29**, panel D). In contrast, the one product detected at sites 8 and 9 (FIG. **29**, panels B and C) established that each of these loci was homozygous. The second individual (FIG. **29**E-F and G-H; Table 13, individual 2) was heterozygous only at sites 3, 5, 6, and 9 and homozygous at sites 1, 2, 4, 7, 8, 10-12. There was a total of 8 differences in the genotypes at these positions between the two persons. Three additional individuals were typed, and all 5 persons had distinct genotypes based on the 12 loci (Table 13).

Although the invention has been described in detail for the purpose of illustration, it is understood that such details are solely for that purpose. The variations can be made therein by those skilled in the art without departing from the spirit of the scope of the invention which is defined by the following claims.

SEQUENCE LISTING

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<400> SEQUENCE: 3

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<210> SEQ ID NO 4
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<400> SEQUENCE: 4

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<210> SEQ ID NO 6
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 6

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cacagtctct cggcgctggg caataataca tagtgcggta gttggc 46

<210> SEQ ID NO 7
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<220> FEATURE:
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<400> SEQUENCE: 7

cacgctatcc cgttagacat cttagtaatt gaggtgcgtg tttgtgcctg tc 52

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<213> ORGANISM: Artificial Sequence
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<400> SEQUENCE: 10

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cgctgccaac taccgcacta tg 22

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agcttcaatg atgagaacct gc 22

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<400> SEQUENCE: 14

gcatagtggt ggctgacctg ttcatat 27

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agcttcaatg atgagaacct gt 22

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ctccatgggc ccagcc 16

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<400> SEQUENCE: 18

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<400> SEQUENCE: 19

ggggacagcc atgcactga 19

<210> SEQ ID NO 20
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gcctctggta gcctttcaac cata 24

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ggggacagcc atgcactgc 19

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<400> SEQUENCE: 23

caccttttag cttcctgagc aatgat 26

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<220> FEATURE:
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ggttgtattt gtcaccatat taatta 26

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Oligonucleotide Sequence

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atggttgtat ttgtcaccat attaattg 28

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<220> FEATURE:
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gggccaagaa ggtatctacc a 21

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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
Oligonucleotide Sequence

<400> SEQUENCE: 29

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Oligonucleotide Sequence

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gggccaagaa ggtatctacc g 21

<210> SEQ ID NO 31
<211> LENGTH: 22

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acacagcagc ttactccaga gg 22

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Oligonucleotide Sequence

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tcaagtccaa ggccattggc ttata 25

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acacagcagc ttactccaga ga 22

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Oligonucleotide Sequence

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ccagcaaaga gaaaagaagg g 21

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Oligonucleotide Sequence

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ccagcaaaga gaaaagaagg a 21

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atgatattag agctcactca tgtccg 26

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Oligonucleotide Sequence

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tgctgtcttc caggaatctg tt 22

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caactctctc gaagccatgt tcacaa 26

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attgctgtct tccaggaatc tgtg 24

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<220> FEATURE:
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<400> SEQUENCE: 43

ggacatagtg accgtgcagg tc 22

<210> SEQ ID NO 44
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<400> SEQUENCE: 44

cttccccagt gtgagtgccg ta 22

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ctatgacacc gtcatcagca gg 22

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<400> SEQUENCE: 47

gacatccagg cccccgac 18

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tactatgaca ccgtcatcag caga 24

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gcgacctgac ttgccgaaga acatgtccca tctcctctac ctgatac 47

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Sequence

<400> SEQUENCE: 55

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<210> SEQ ID NO 73
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<400> SEQUENCE: 73

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ggagcacgct atcccgttag ac 22

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cgctgccaac taccgcacat g 21

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cctcgtgcga ggcgtattct g 21

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gcgacctgac ttgccgaaga ac 22

What is claimed is:

1. A method for identifying a target nucleotide sequence comprising:

forming a ligation product on a target nucleotide sequence in a ligation detection reaction mixture, wherein the ligation product comprises an upstream primer portion and a downstream primer portion, wherein the upstream primer portion and the downstream primer portion are not complementary with the target nucleotide sequence;

amplifying the ligation product to form an amplified ligation product in a polymerase chain reaction (PCR) mixture, wherein the PCR mixture comprises an upstream primer and a downstream primer; and

detecting the amplified ligation product to identify the target nucleotide sequence.

2. The method according to claim **1** wherein the ligation detection reaction mixture comprises adjacently hybridizing ligation probes.

3. The method according to claim **1** wherein the ligation product results from the ligation of two ligation probes, wherein the two ligation probes are two different molecules.

4. The method according to claim **1**, wherein the upstream primer or the downstream primer contains a label.

5. The method according to claim **1**, wherein the ligation product can be distinguished from other nucleic acids in the PCR, said method further comprising:

providing a solid support with different capture oligonucleotides immobilized at different particular sites, wherein the capture oligonucleotides have nucleotide sequences complementary to a unique nucleotide sequence of an extension product.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

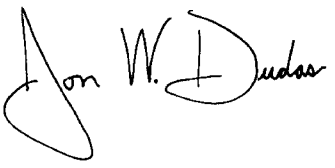
PATENT NO. : 7,312,039 B2
APPLICATION NO. : 11/590056
DATED : December 25, 2007
INVENTOR(S) : Barany et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Col. 1 at lines 20-21, delete "This invention was developed with government funding under National lnstitutes of Health Grant No. GM41337-06. The U.S. Government may retain certain rights." and insert --This invention was made with government support under grant GM41337-06 awarded by the National lnstitutes of Health. The government has certain rights in the invention-- in its place.

Signed and Sealed this

Fourteenth Day of October, 2008



JON W. DUDAS
Director of the United States Patent and Trademark Office