Exhibit I

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

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See application file for complete search history.

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(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.

46 Claims, 34 Drawing Sheets

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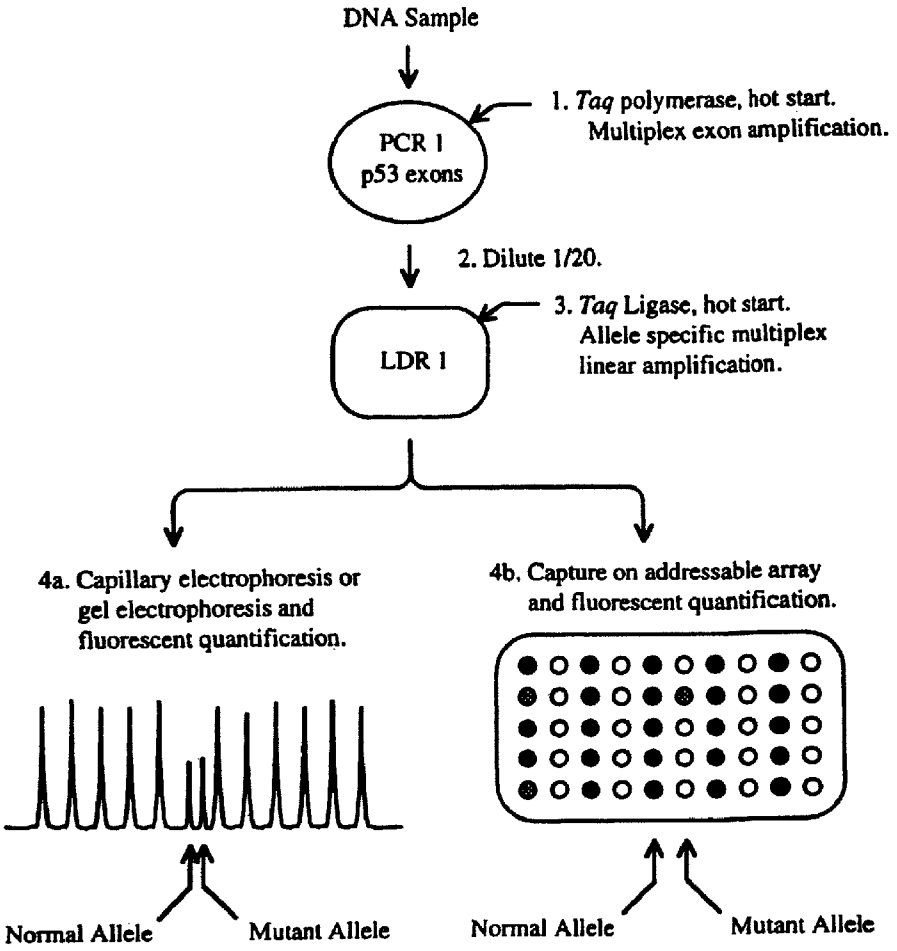

DNA Sample
1. Taq polymerase, hot start.
Multiplex exon amplification.
PCR 1
p53 exons
2. Dilute 1/20.
3. Taq Ligase, hot start.
Allele specific multiplex
linear amplification.
LDR 1
4a. Capillary electrophoresis or
gel electrophoresis and
fluorescent quantification.
4b. Capture on addressable array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

FIG. 1

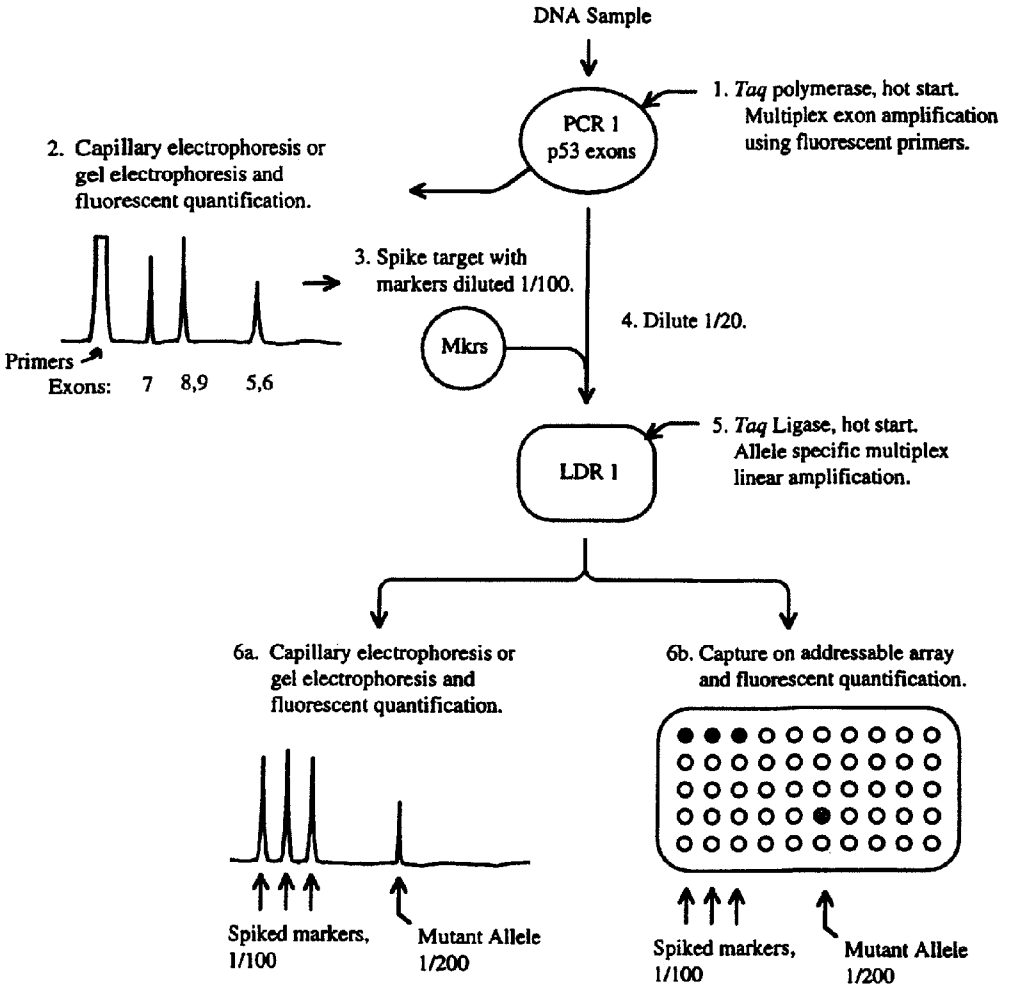

DNA Sample
1. Taq polymerase, hot start. Multiplex exon amplification using fluorescent primers.
PCR 1 p53 exons
2. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
3. Spike target with markers diluted 1/100.
Mkrs
4. Dilute 1/20.
Primers
Exons: 7 8,9 5,6
5. Taq Ligase, 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

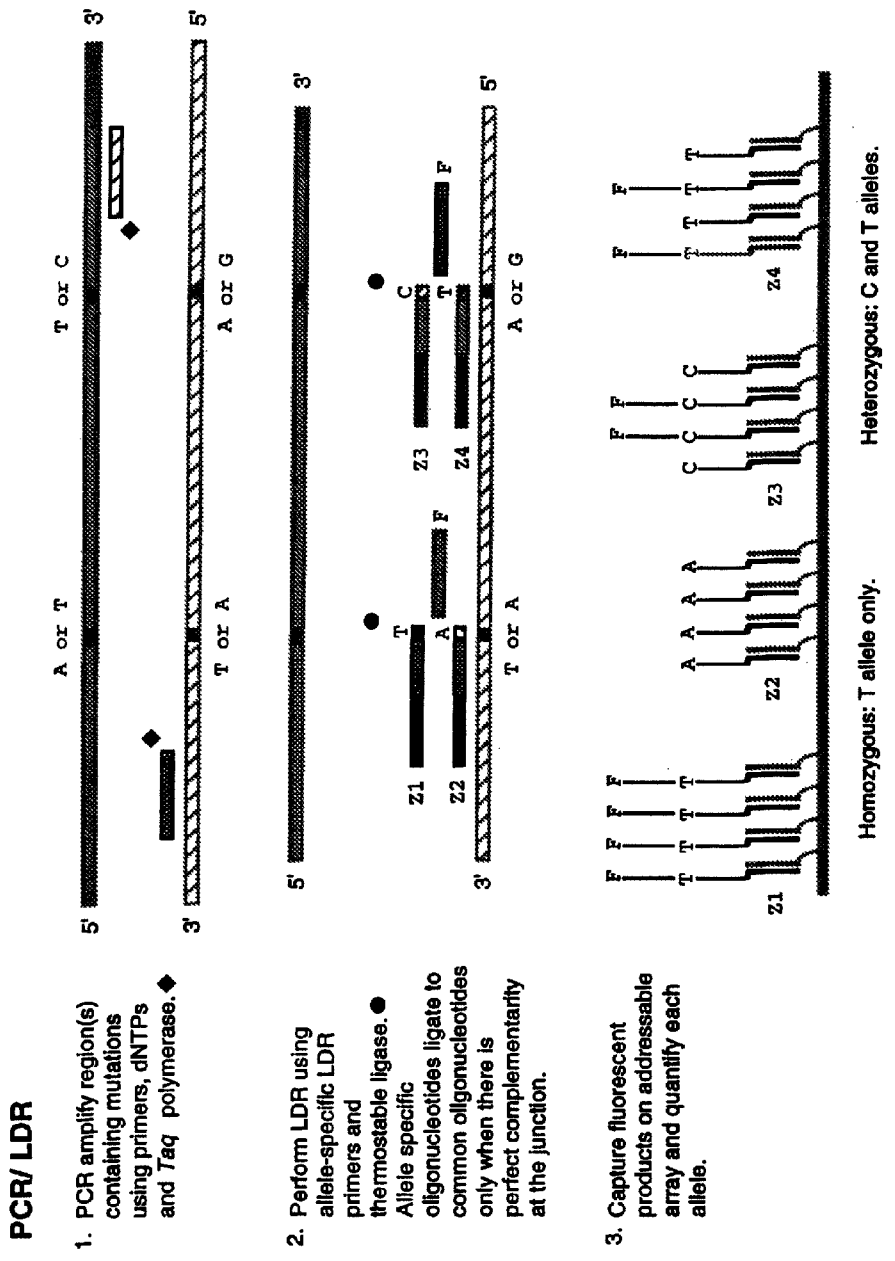

PCR/ LDR
1. PCR amplify region(s) containing mutations using primers, dNTPs and *Taq* polymerase. ◆
2. Perform LDR using allele-specific LDR primers and thermostable ligase. ● Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
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
Homozygous: T allele only.
Heterozygous: C and T alleles.

FIG. 3

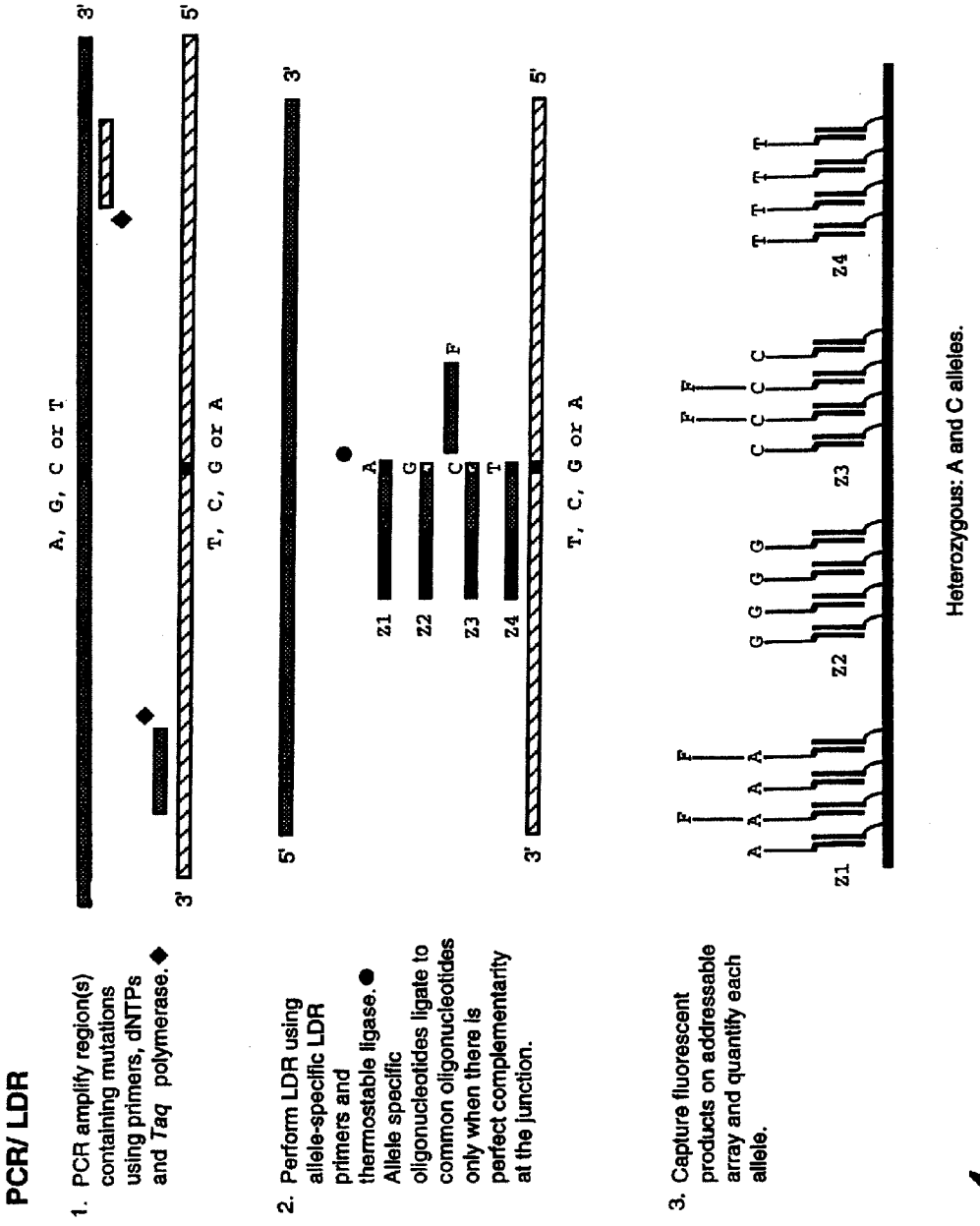

PCR/ LDR
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
A, G, C or T
T, C, G or A
3'
5'
2. Perform LDR using allele-specific LDR primers and thermostable ligase.
Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity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

FIG. 4

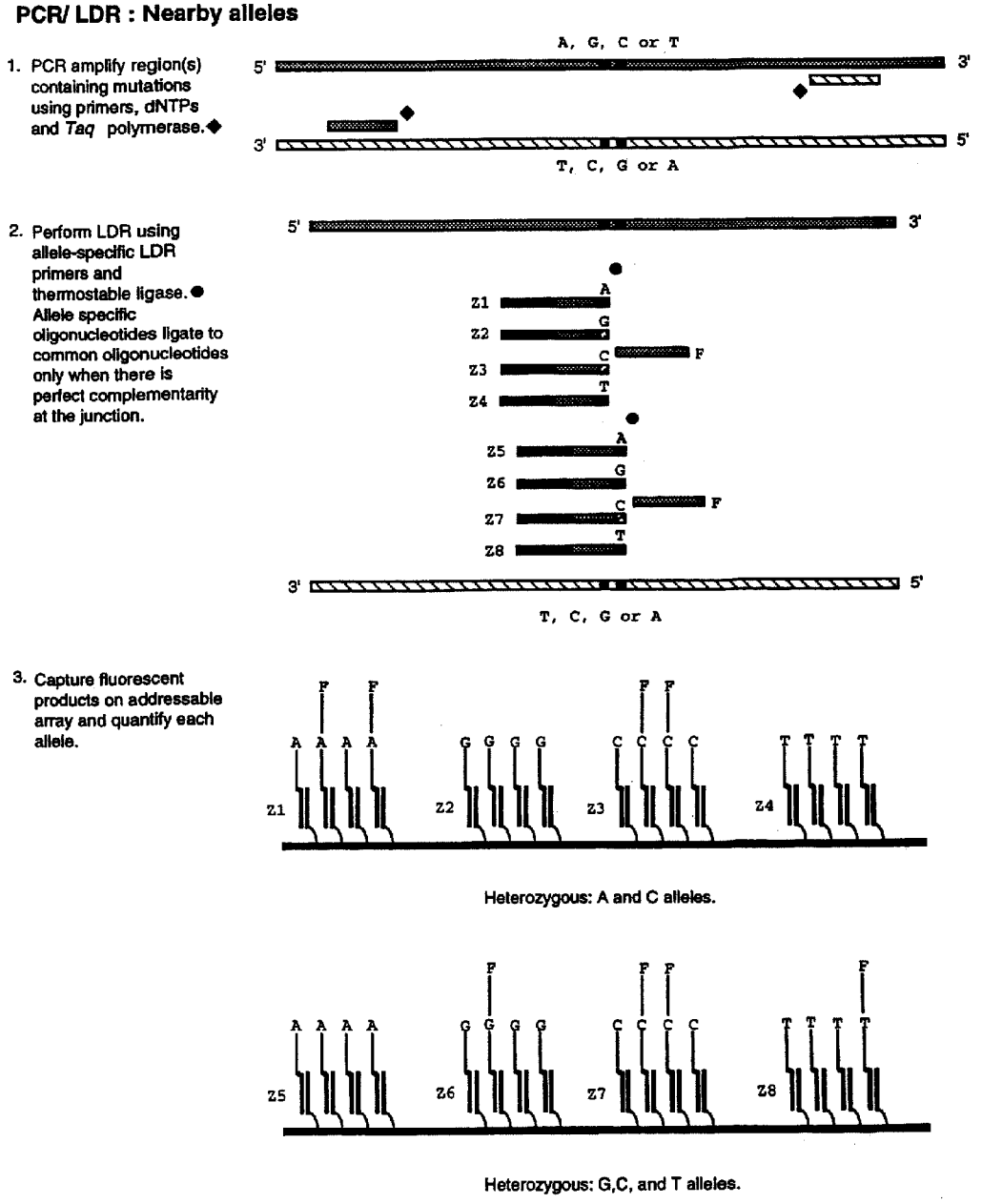

PCR/ LDR : Nearby alleles
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
A, G, C or T
5'
3'
3'
5'
T, C, G or A
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common oligonucleotides only when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

FIG. 5

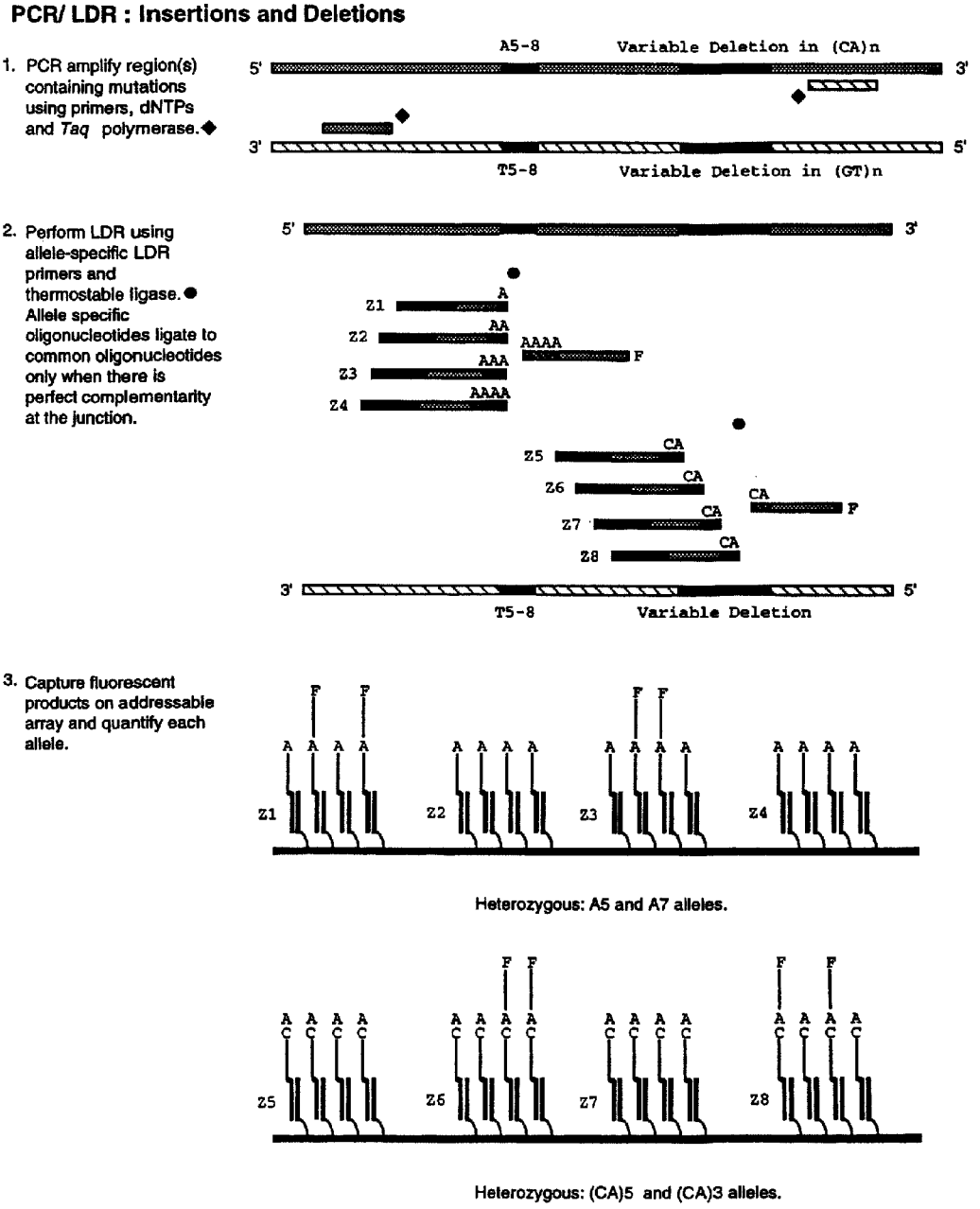

PCR/ LDR : Insertions and Deletions
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
A5-8
Variable Deletion in (CA)n
5'
3'
T5-8
Variable Deletion in (GT)n
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate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

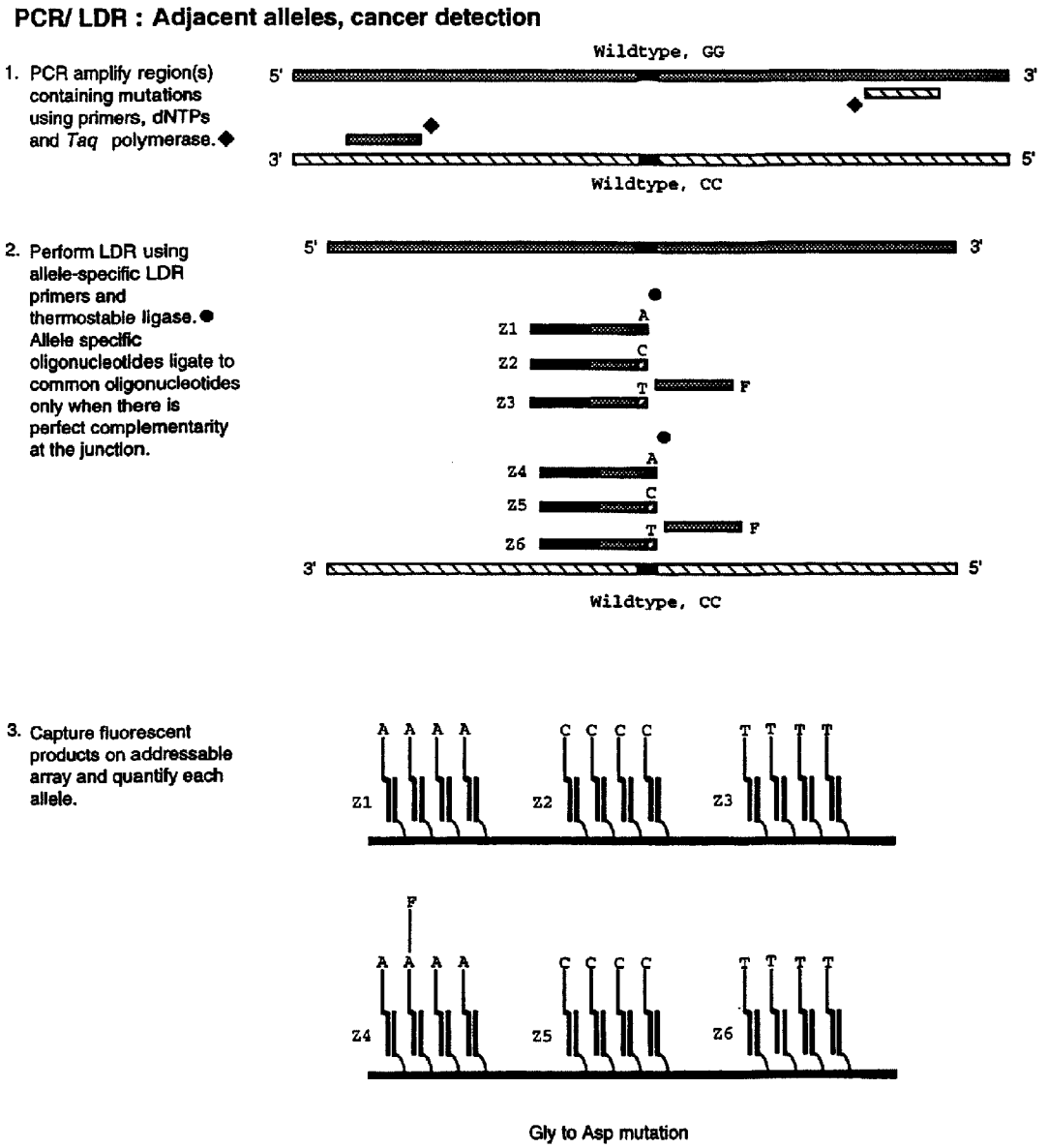

PCR/ LDR : Adjacent alleles, cancer detection
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
Wildtype, GG
5'
3'
3'
5'
Wildtype, CC
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate to common oligonucleotides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

FIG. 7

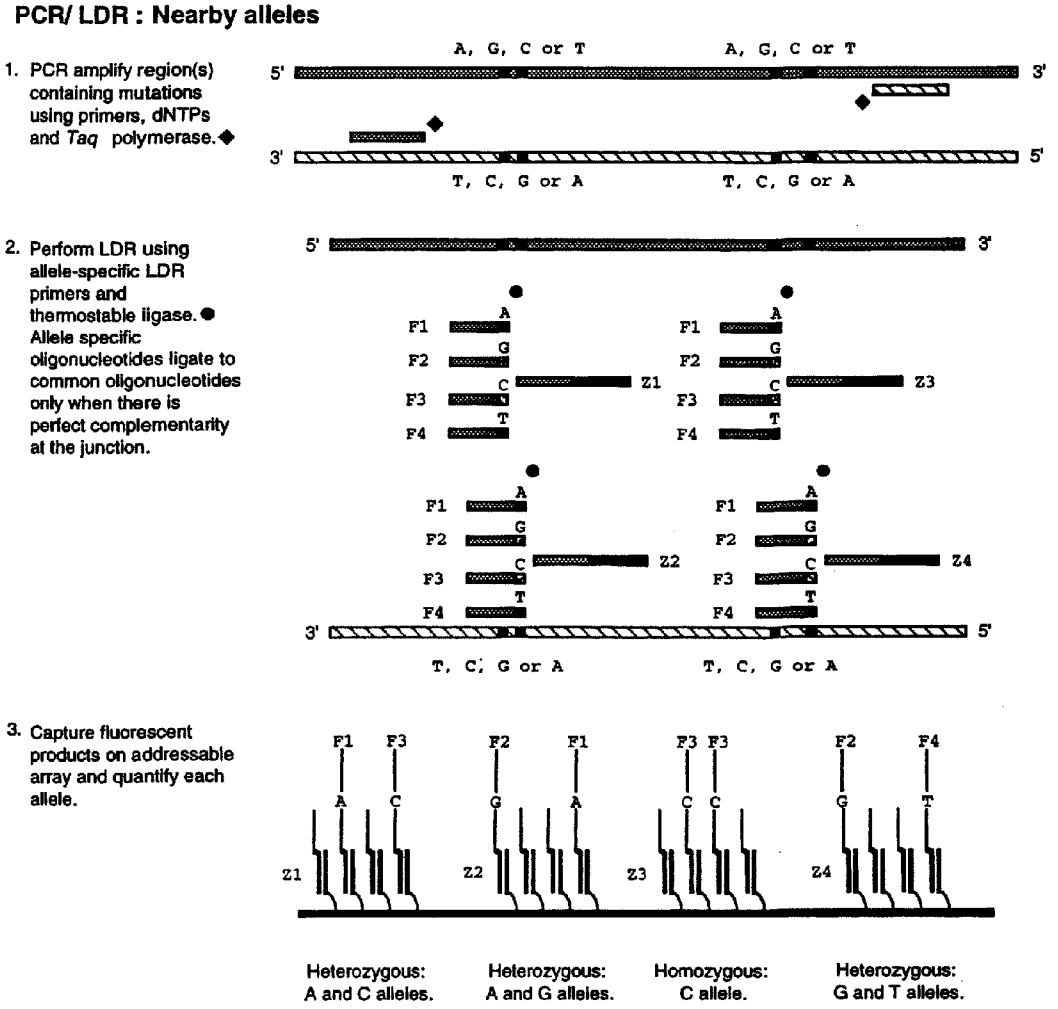

PCR/ LDR : Nearby alleles
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the 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

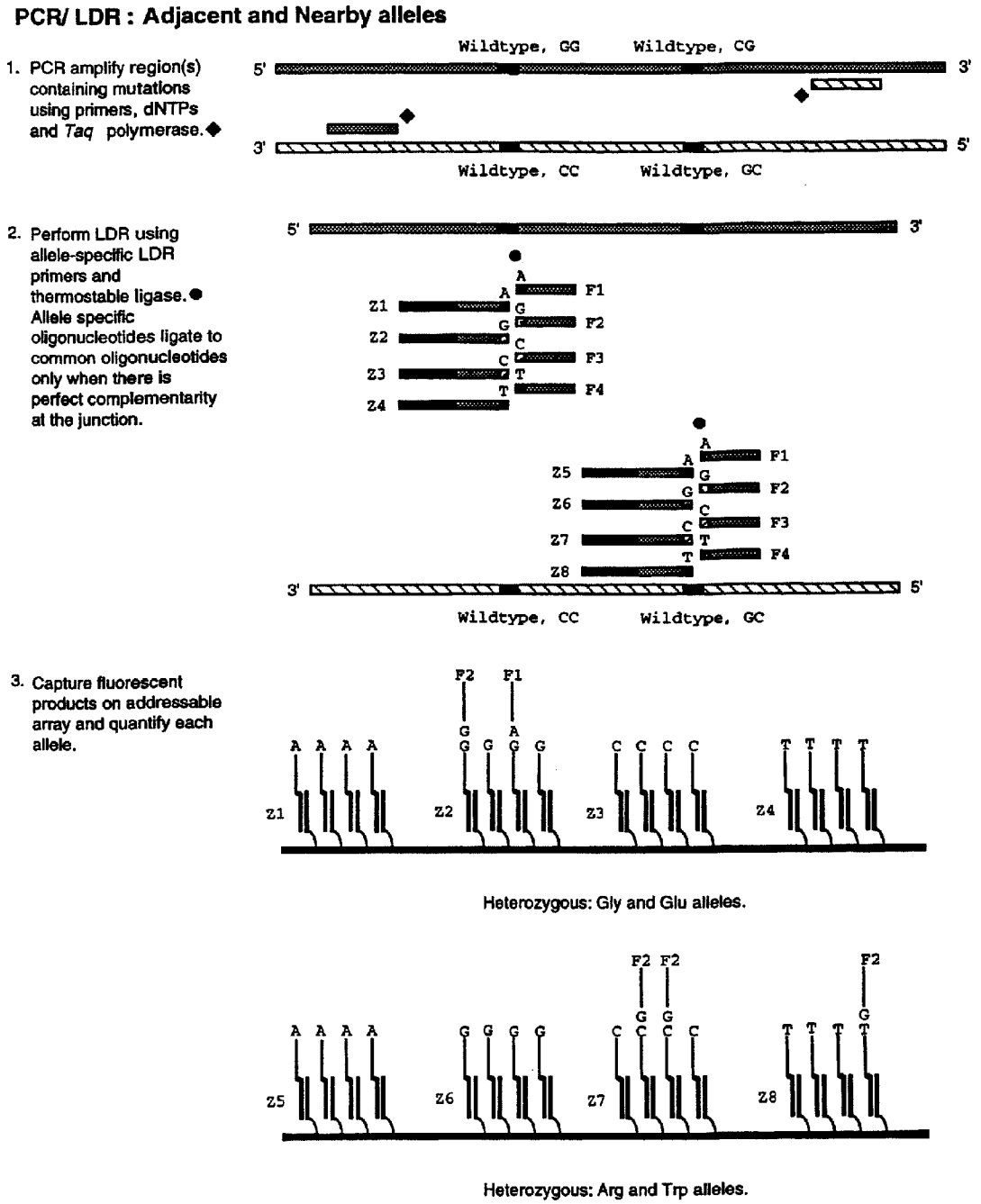

PCR/ LDR : Adjacent and Nearby alleles
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
Wildtype, GG
Wildtype, CG
Wildtype, CC
Wildtype, GC
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate to common 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

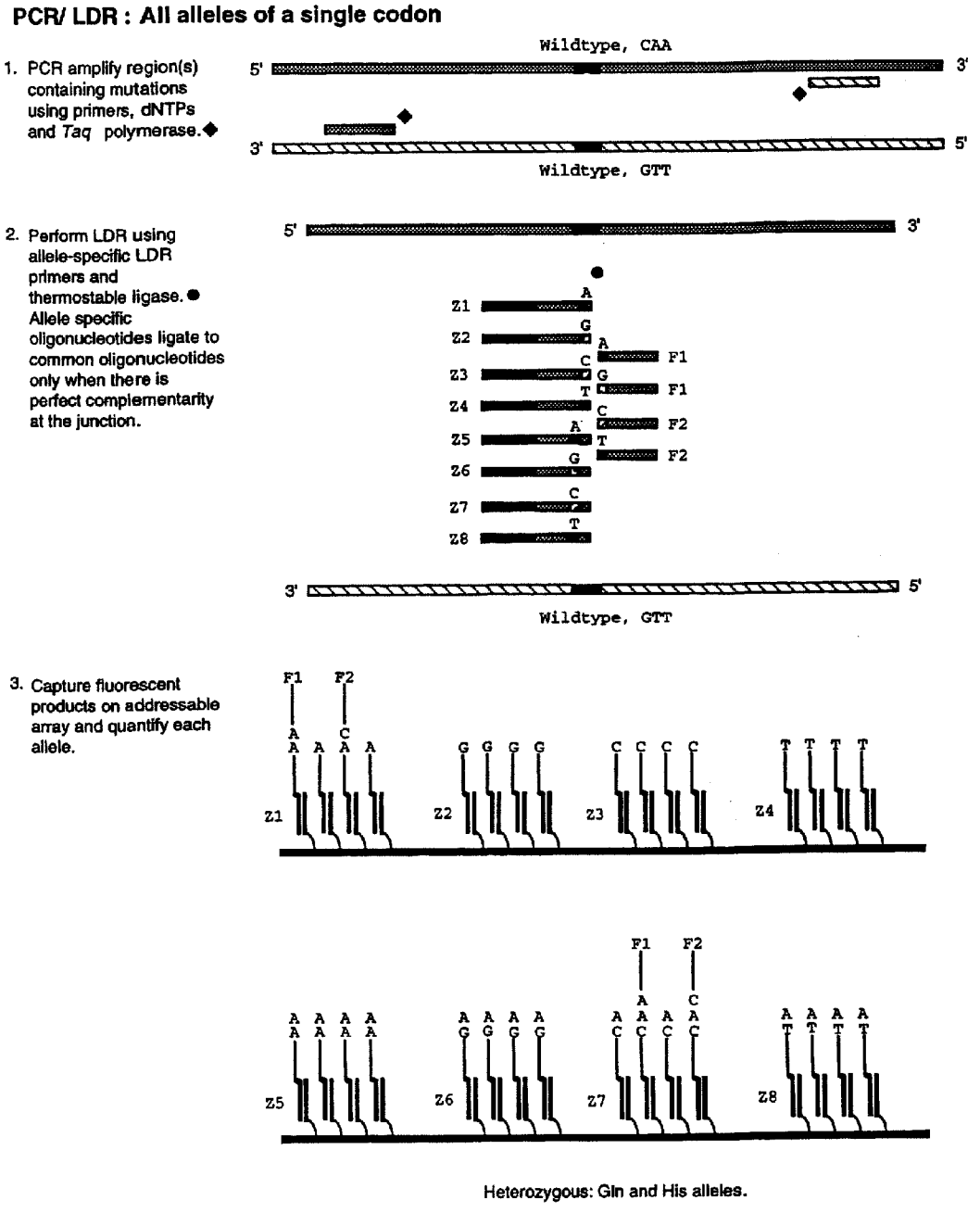
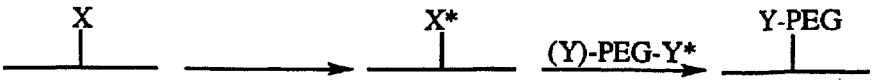

PCR/ LDR : All alleles of a single codon
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
Wildtype, CAA
5'
3'
3'
5'
Wildtype, GTT
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common oligonucleotides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

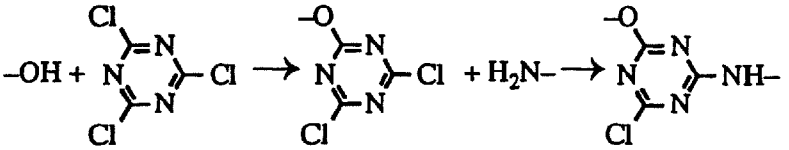
W = protecting group, e.g. Boc, Fmoc
Z = activating group, e.g. imidazole (Im), *p*-nitrophenol (OPnp), hydroxysuccinimide (OSu), pentafluorophenol (OPfp)
PEG = oligo or poly(ethylene glycol), backbone $(CH_2CH_2O)_n$ n = 6 to 200 (can also be grown by anionic polymerization with)
WSC = water soluble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

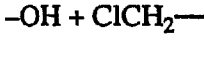
Functional group transformations/activation (as needed), X → X*, Y → Y*

–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– ⟶ –O(C=O)NH–



–OH + O=C=N– ⟶ –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 ⟶ $-OCH_2(C{=}O)H + H_2N- + NaCNBH_3 \longrightarrow -OCH_2CH_2NH-$

FIG. 11

FIG. 12A

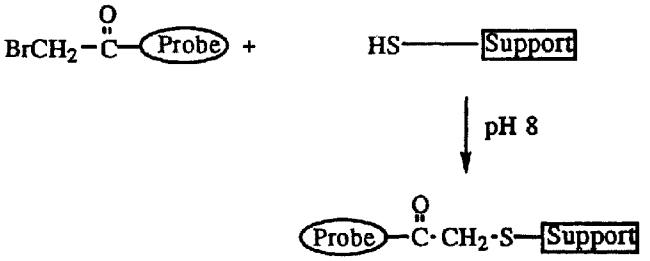


FIG. 12B

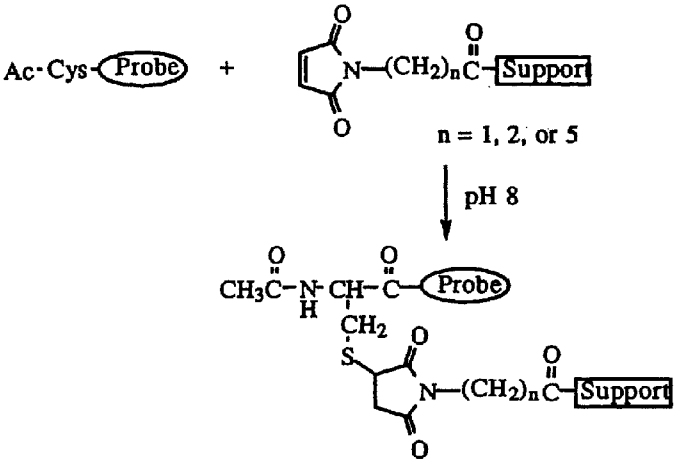
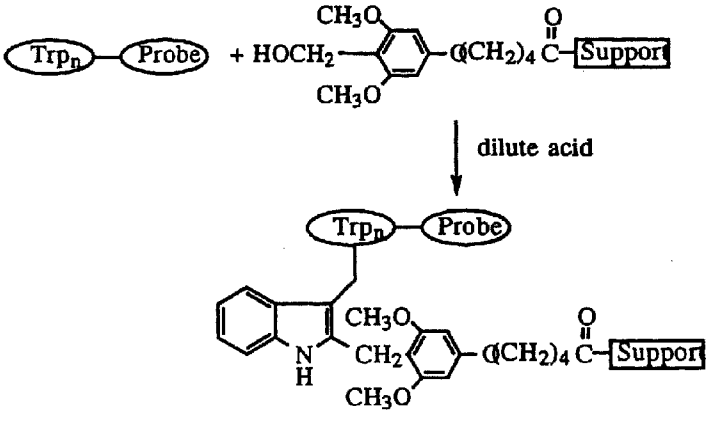


FIG. 12C



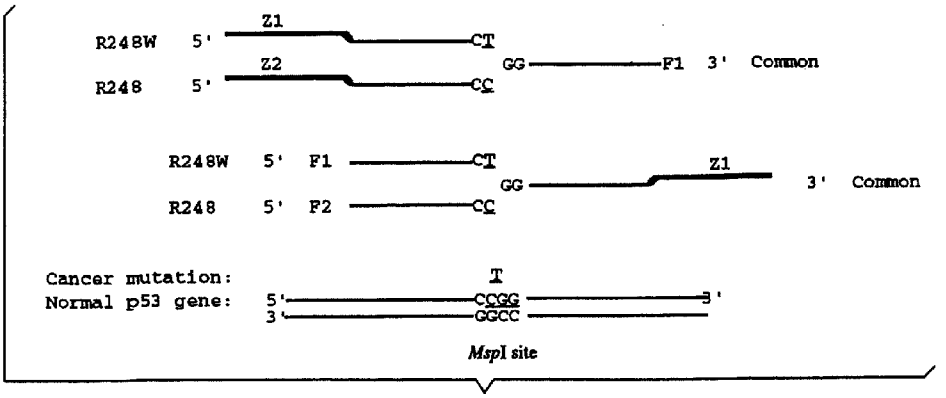


FIG. 13A

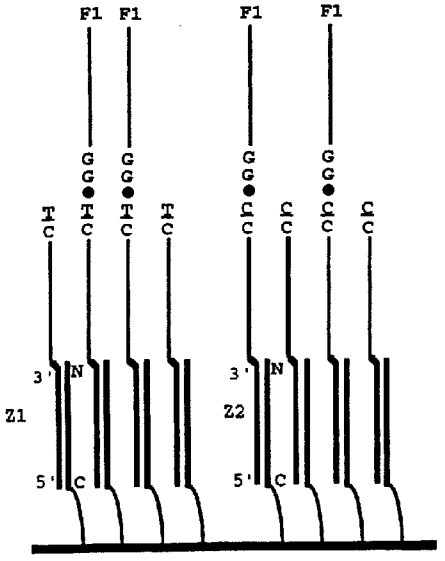


FIG. 13B

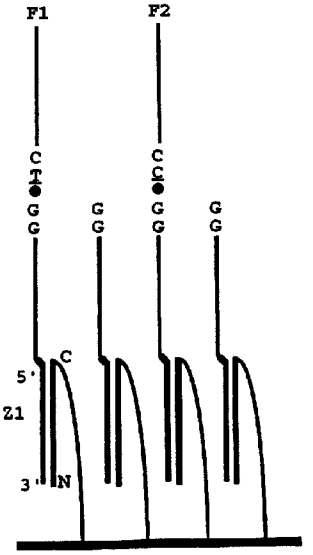


FIG. 13C

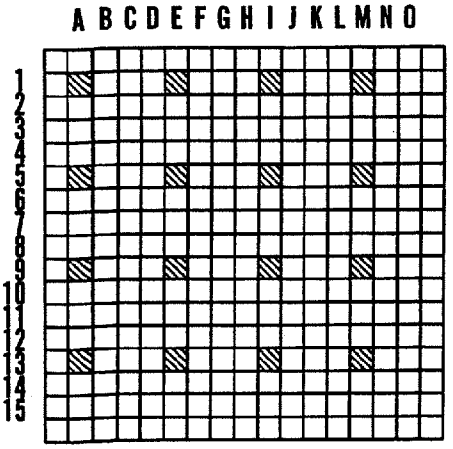


FIG. 14A

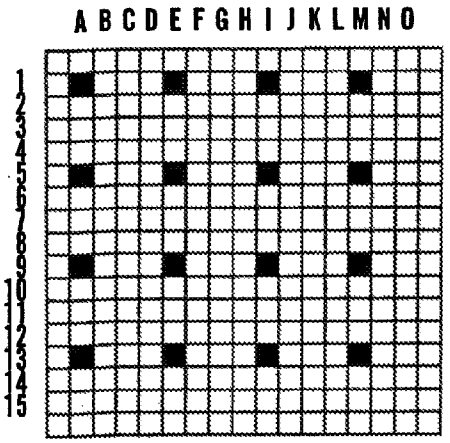


FIG. 14B

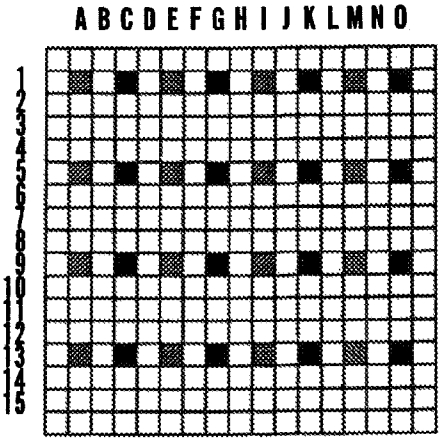


FIG. 14C

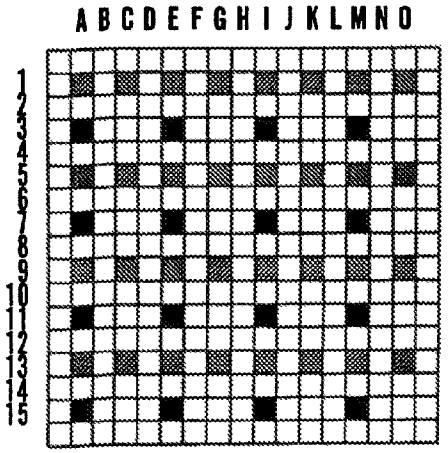


FIG. 14D

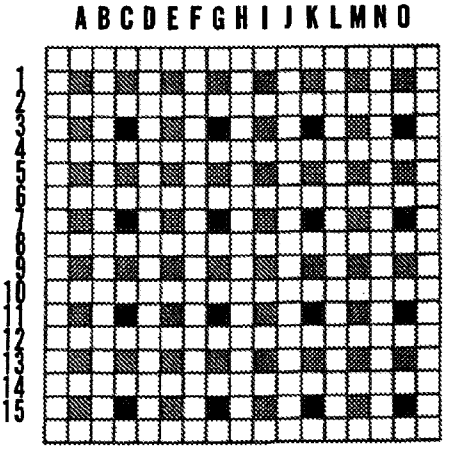


FIG. 14E

FIG. 15A

1st addition of unique 24mers.

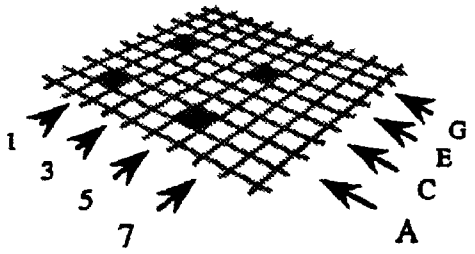


FIG. 15B

2nd addition of unique 24mers.

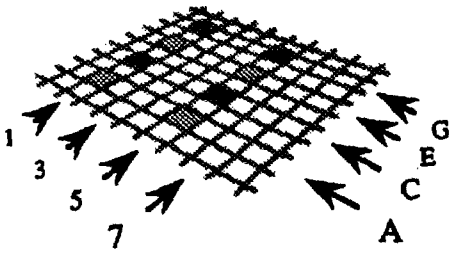


FIG. 15C

3rd addition of unique 24mers.

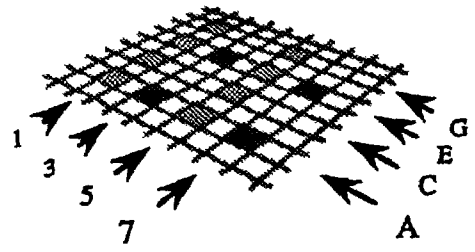
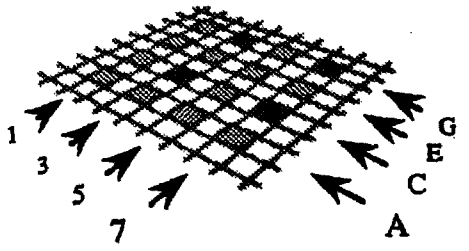


FIG. 15D

4th addition of unique 24mers.



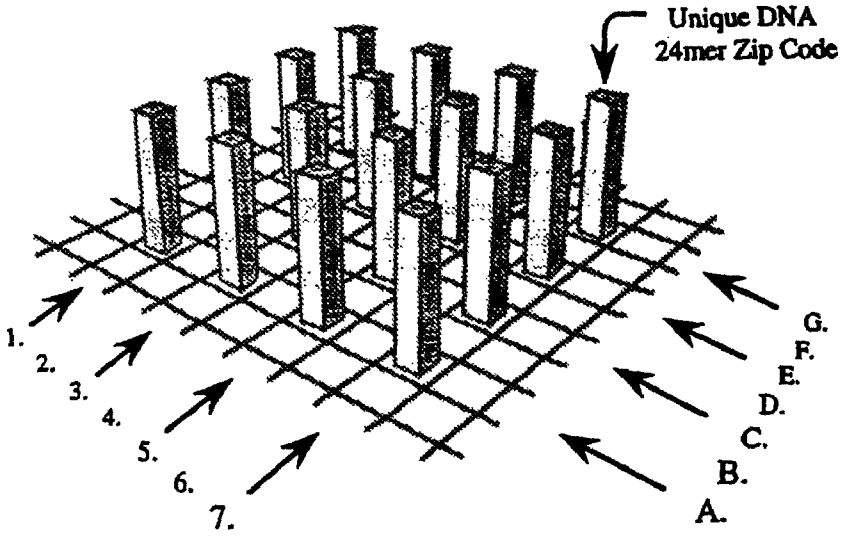


FIG. 15E

FIG. 16A

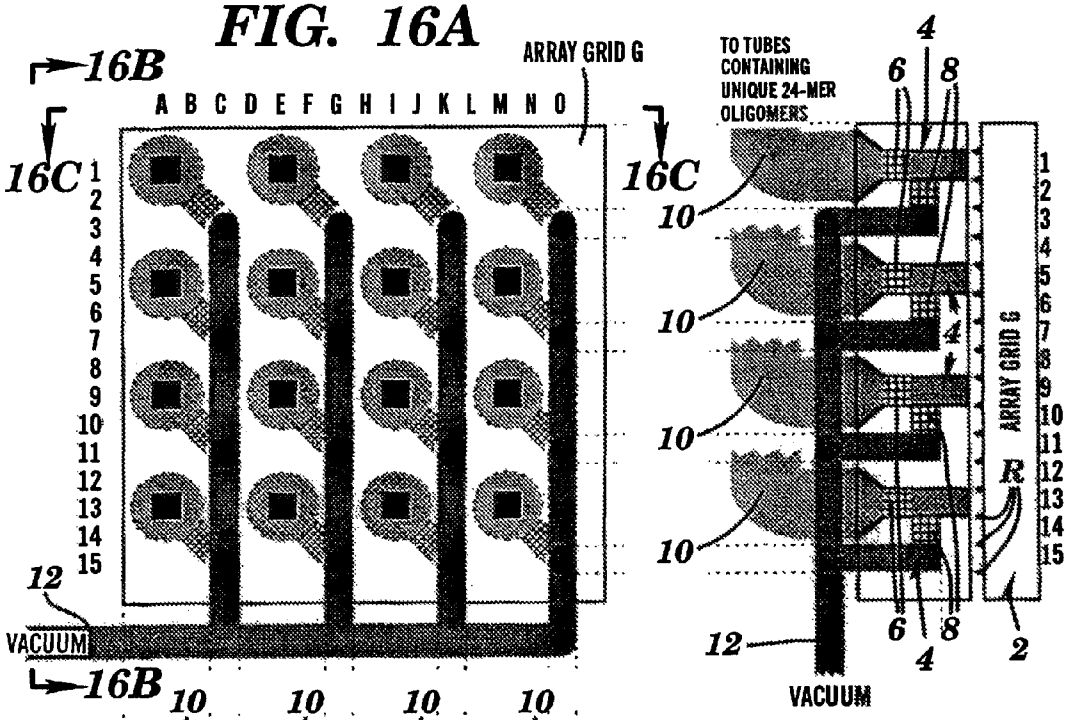


FIG. 16B

FIG. 16C

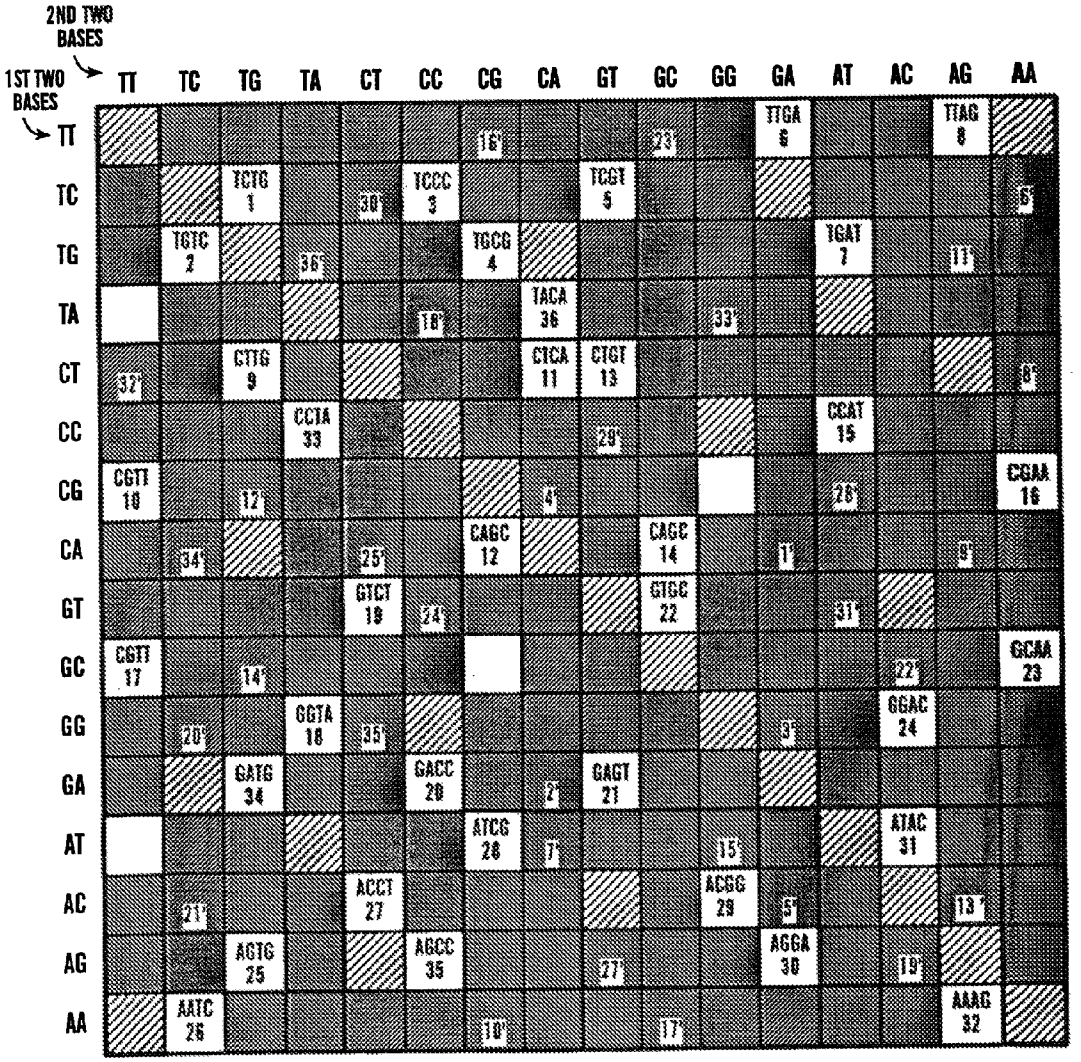

2ND TWO BASES
1ST TWO BASES
TT TC TG TA CT CC CG CA GT GC GG GA AT AC AG AA
TTGA 6
TTAG 8
TCTG 1
TCCC 3
TCGT 5
TGTC 2
TGCG 4
TGAT 7
TACA 36
CTTG 9
CTCA 11
CTGT 13
CCTA 33
CCAT 15
CGTT 18
CGAA 16
CAGC 12
CAGC 14
GTCT 19
GTGC 22
CGTT 17
GCAA 23
GGTA 18
GGAC 24
GATG 34
GACC 28
GAGT 21
ATCG 28
ATAC 31
ACCT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

FIG. 17

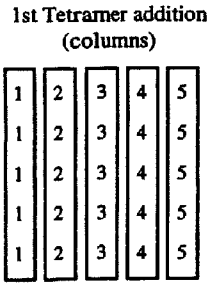


FIG. 18A

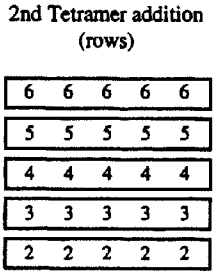


FIG. 18B

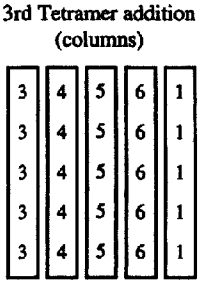


FIG. 18C

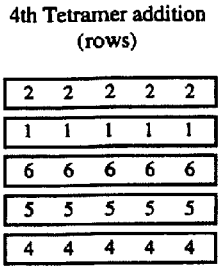


FIG. 18D

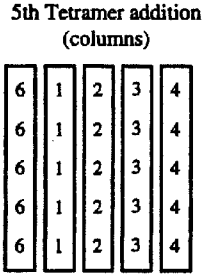


FIG. 18E

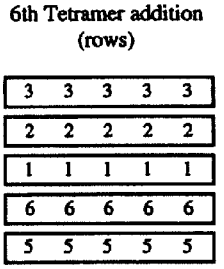


FIG. 18F

Addressable array with full length PNA 24mers

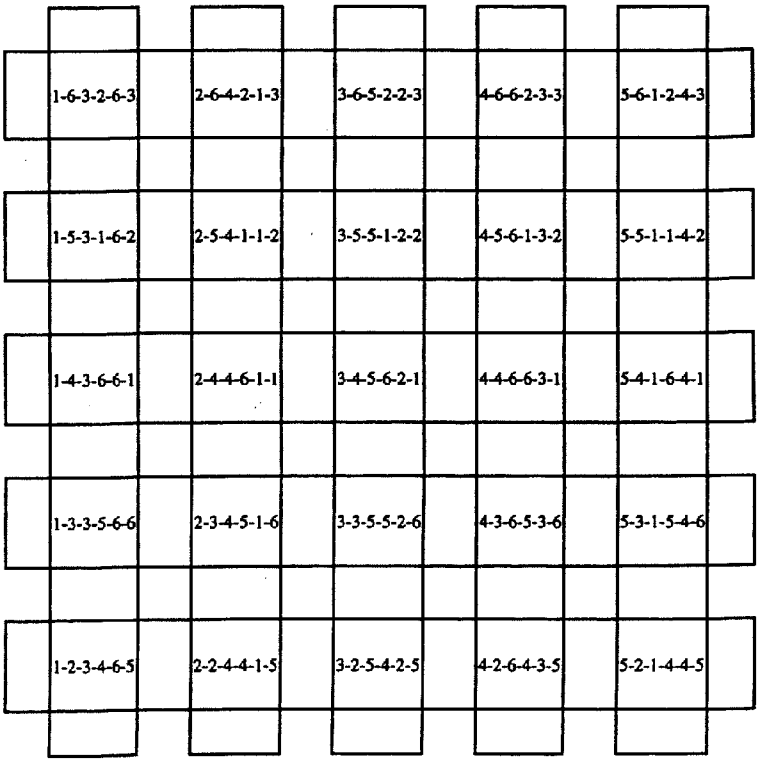


FIG. 18G

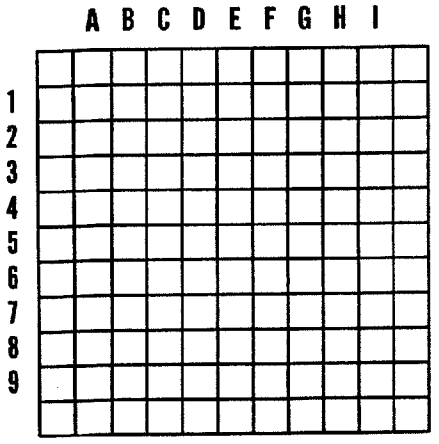


FIG. 19A

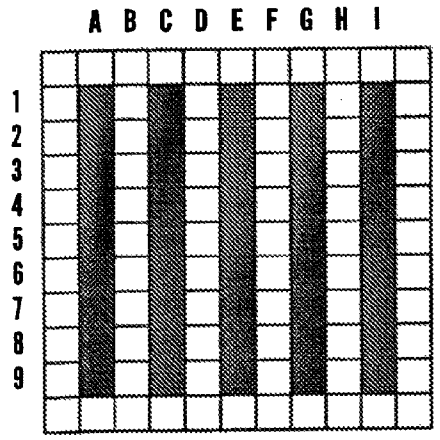


FIG. 19B

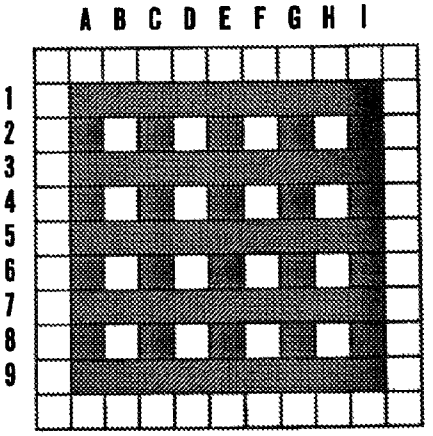


FIG. 19C

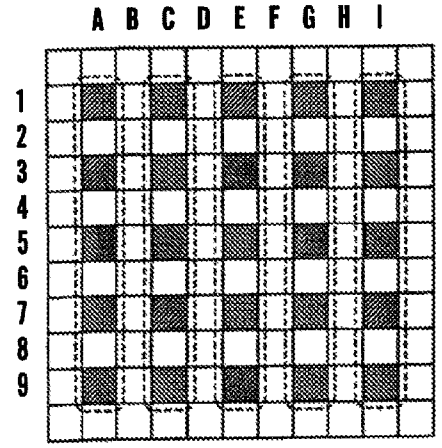


FIG. 19D

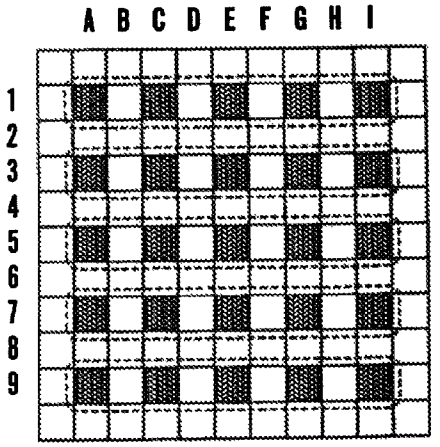


FIG. 19E

FIG. 20A

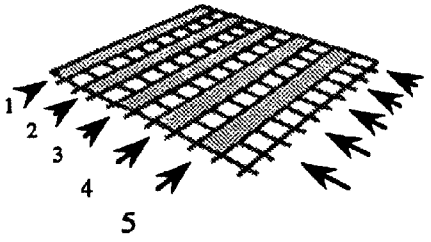
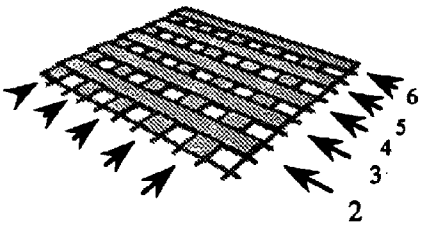


FIG. 20B



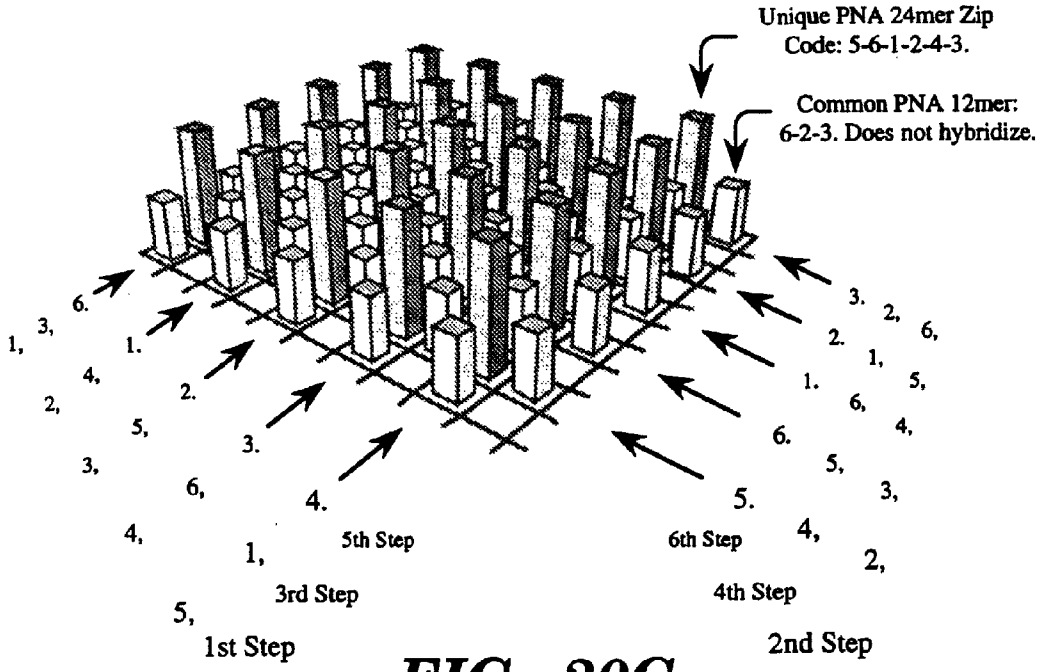


FIG. 20C

FIG. 21A



FIG. 21B



FIG. 21C

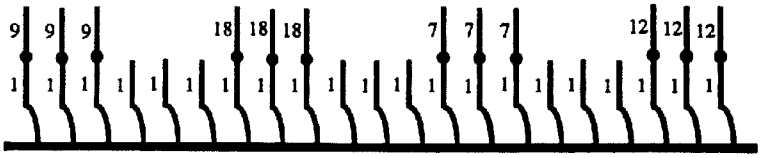


FIG. 21D

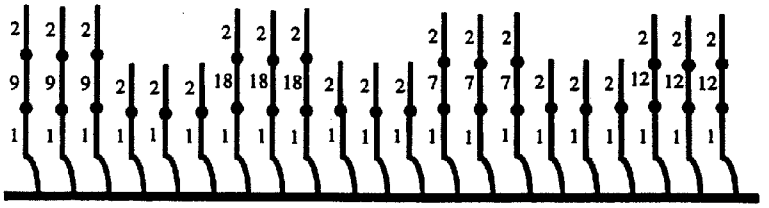


FIG. 21E

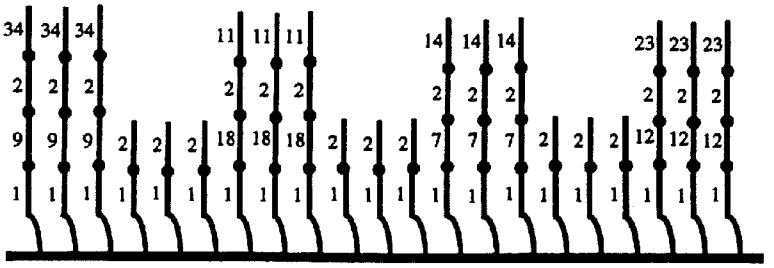
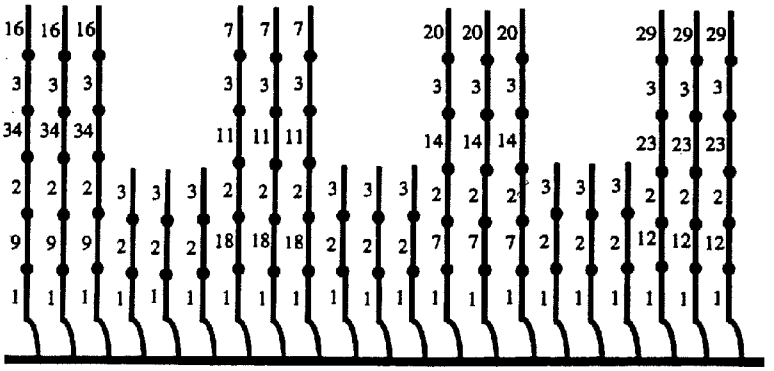


FIG. 21F



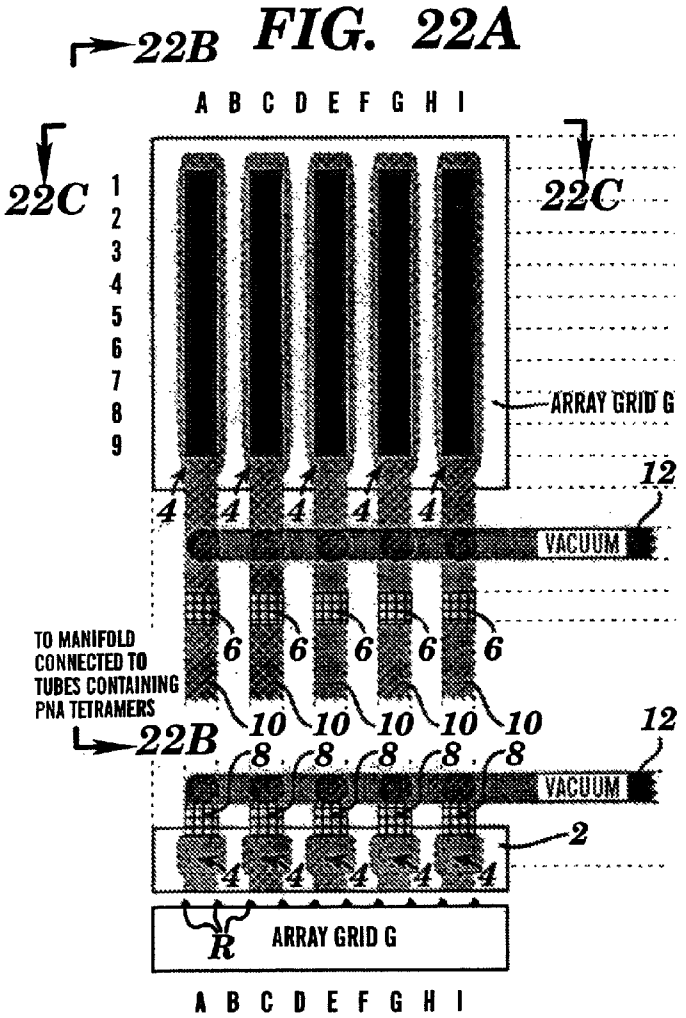


FIG. 22A

FIG. 22C

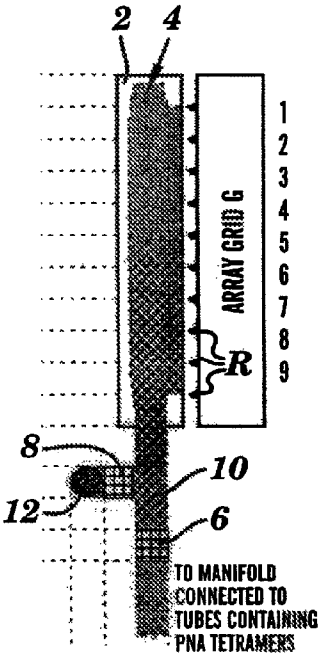


FIG. 22B

FIG. 23A

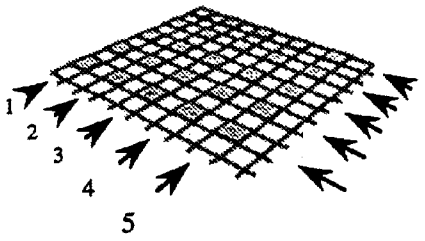
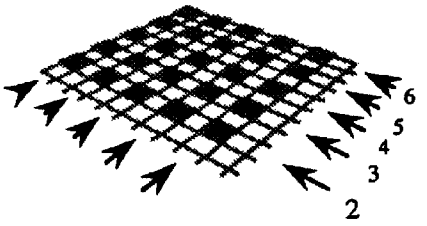


FIG. 23B



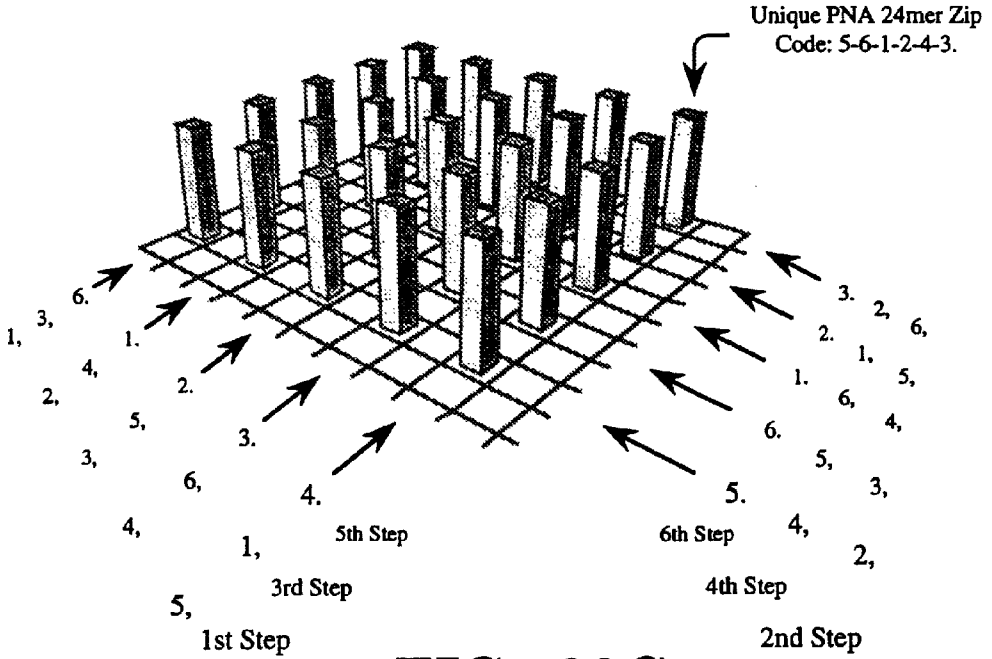


FIG. 23C

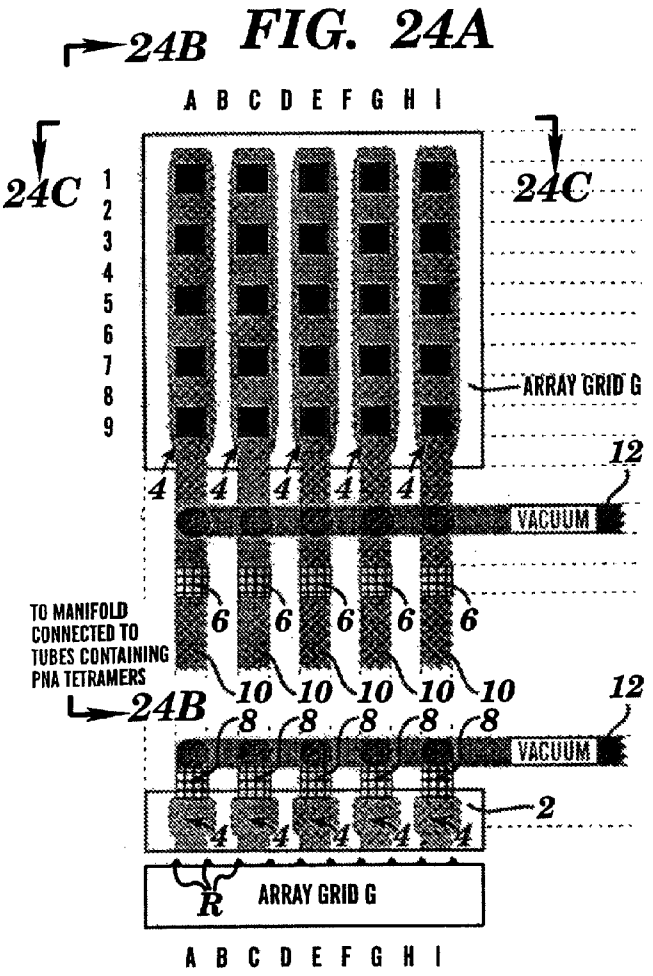


FIG. 24A

FIG. 24C

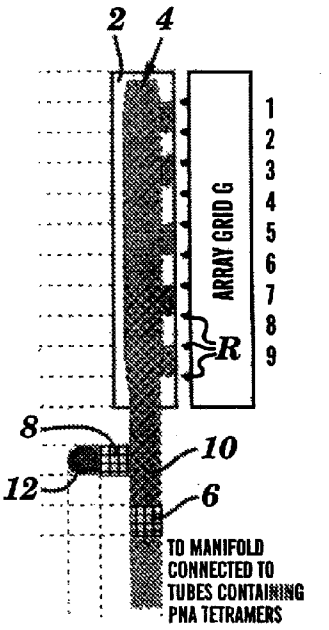


FIG. 24B

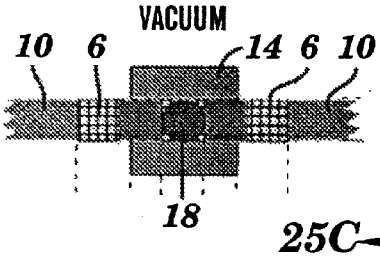


FIG. 25B

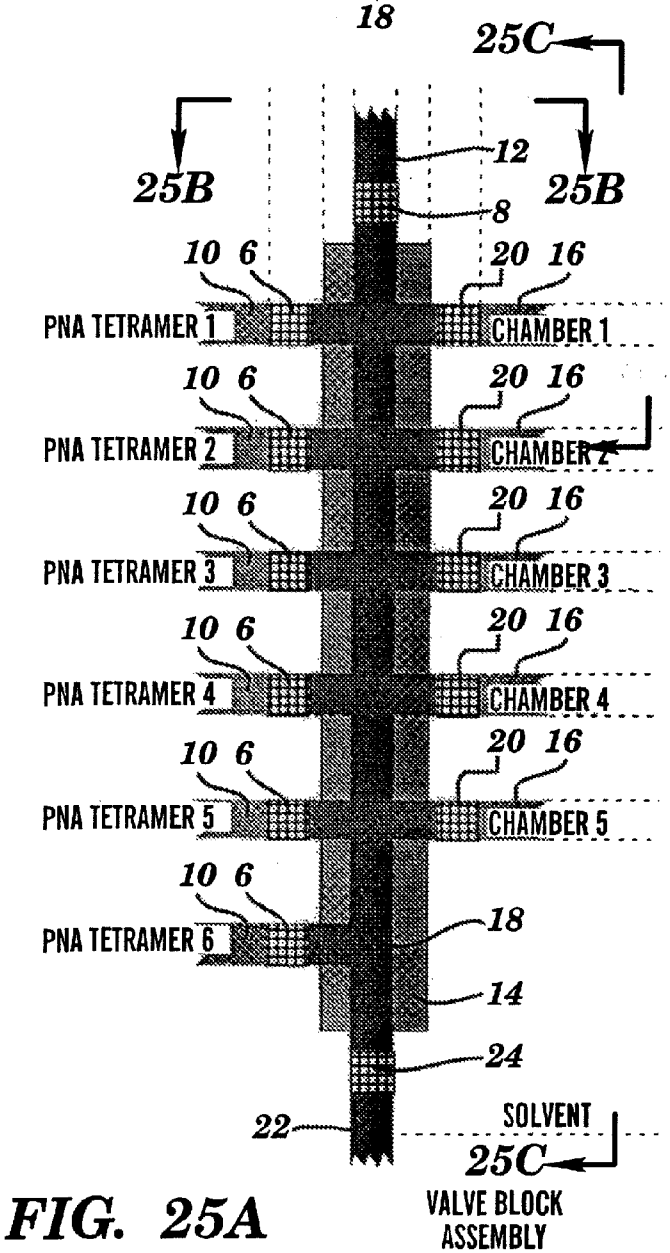


FIG. 25A

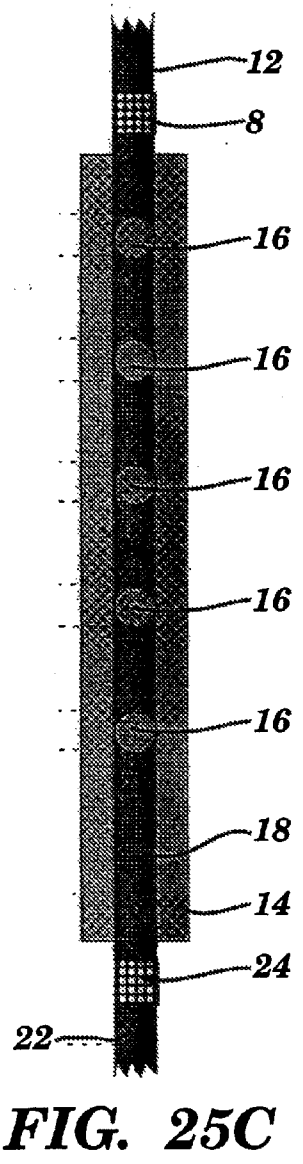


FIG. 25C

6 INPUTS AND 5 OUTPUTS

FIG. 26A

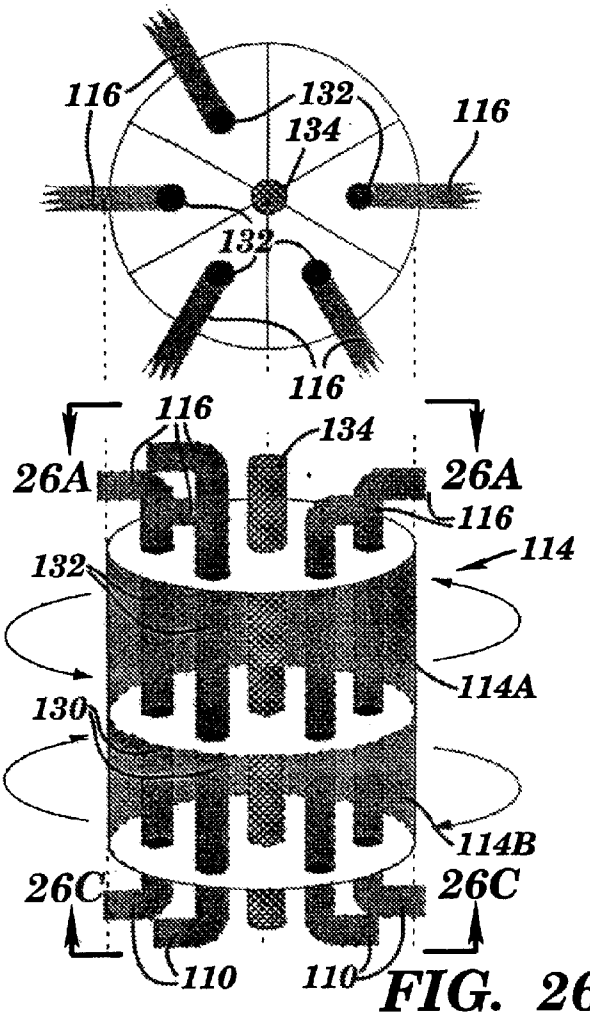


FIG. 26B

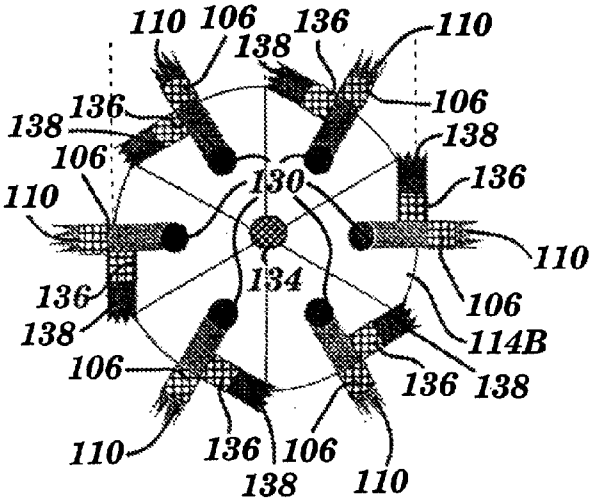


FIG. 26C

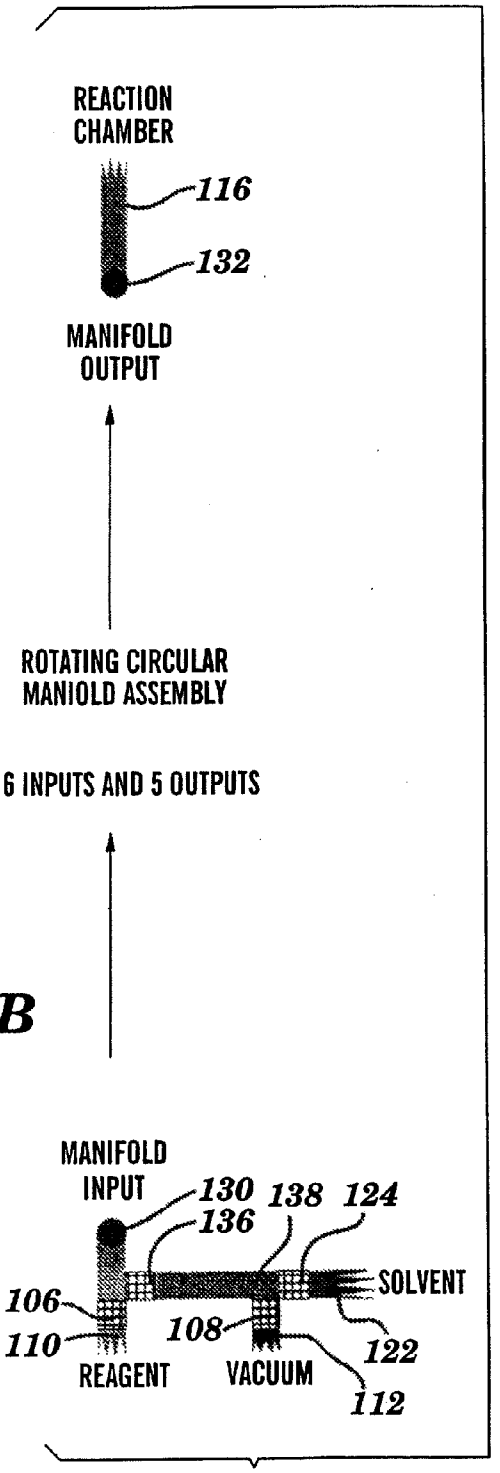


FIG. 26D

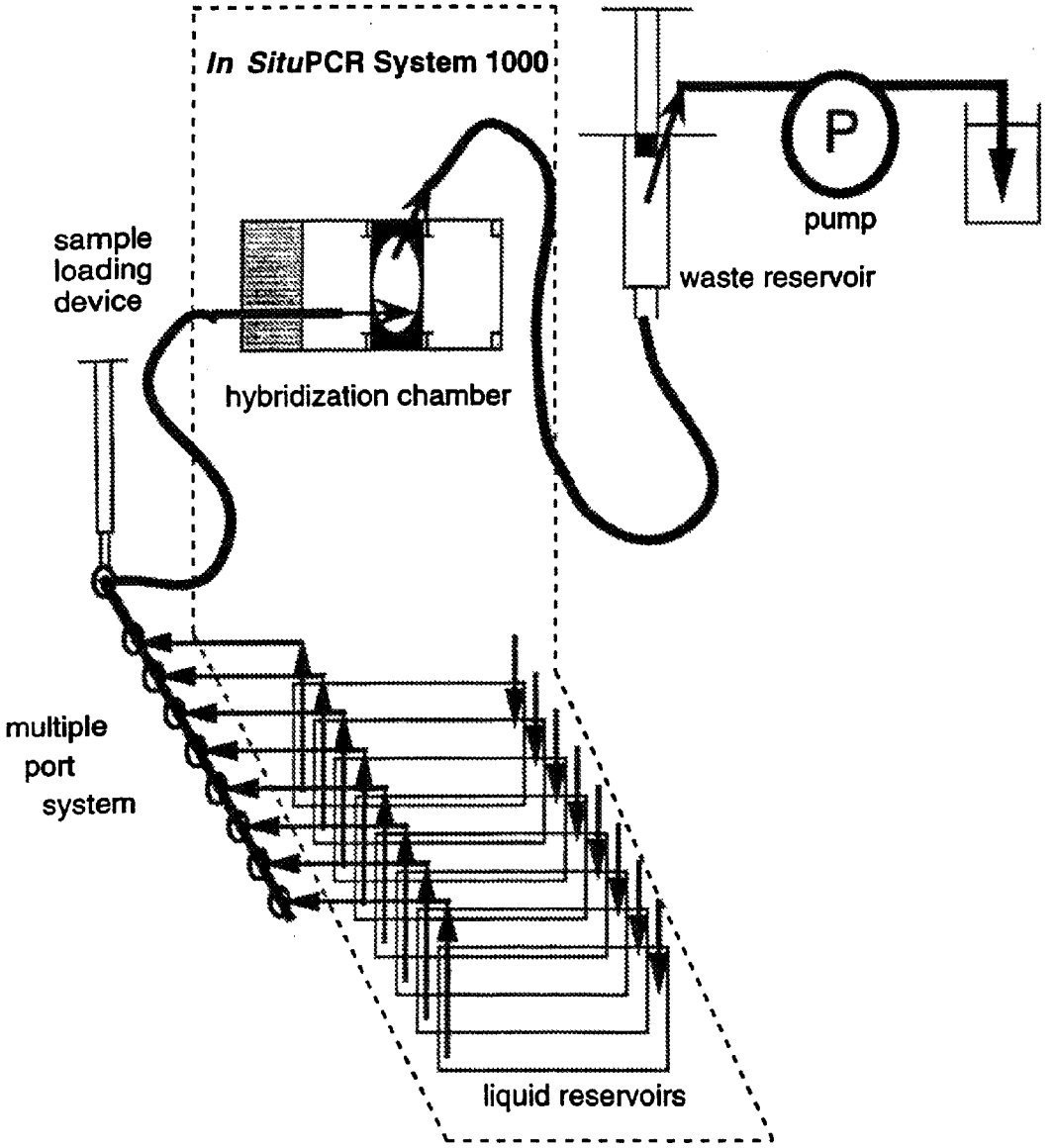

In SituPCR System 1000
P
pump
waste reservoir
sample
loading
device
hybridization chamber
multiple
port
system
liquid reservoirs

FIG. 27

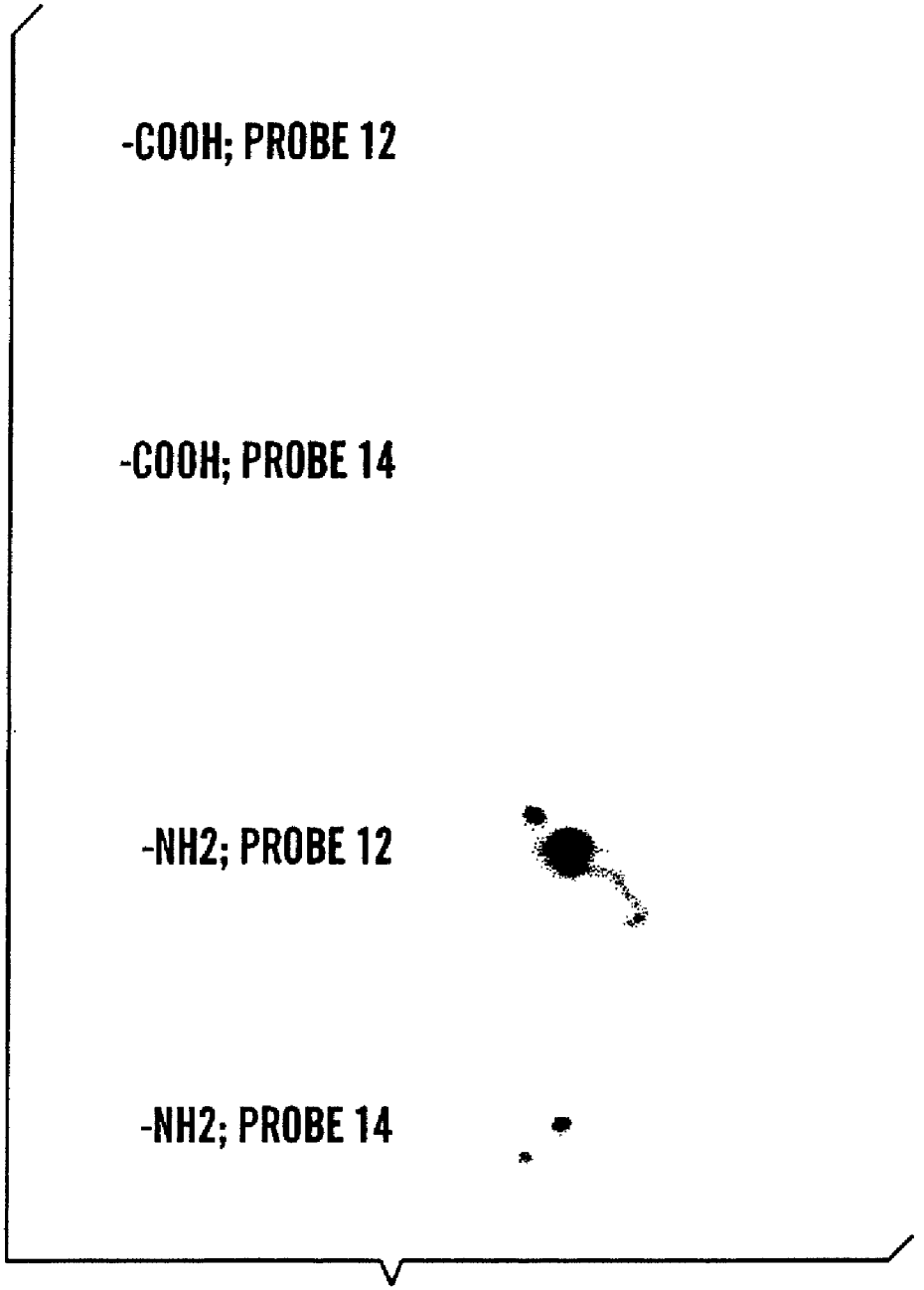


FIG. 28

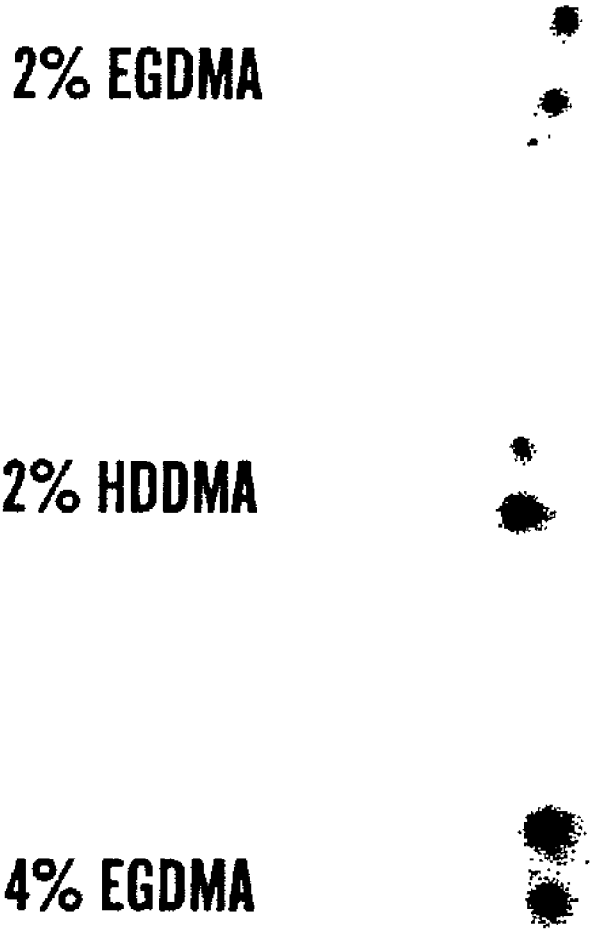


FIG. 29

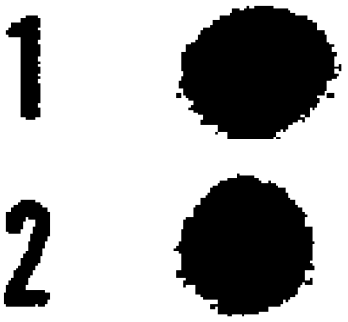


FIG. 30

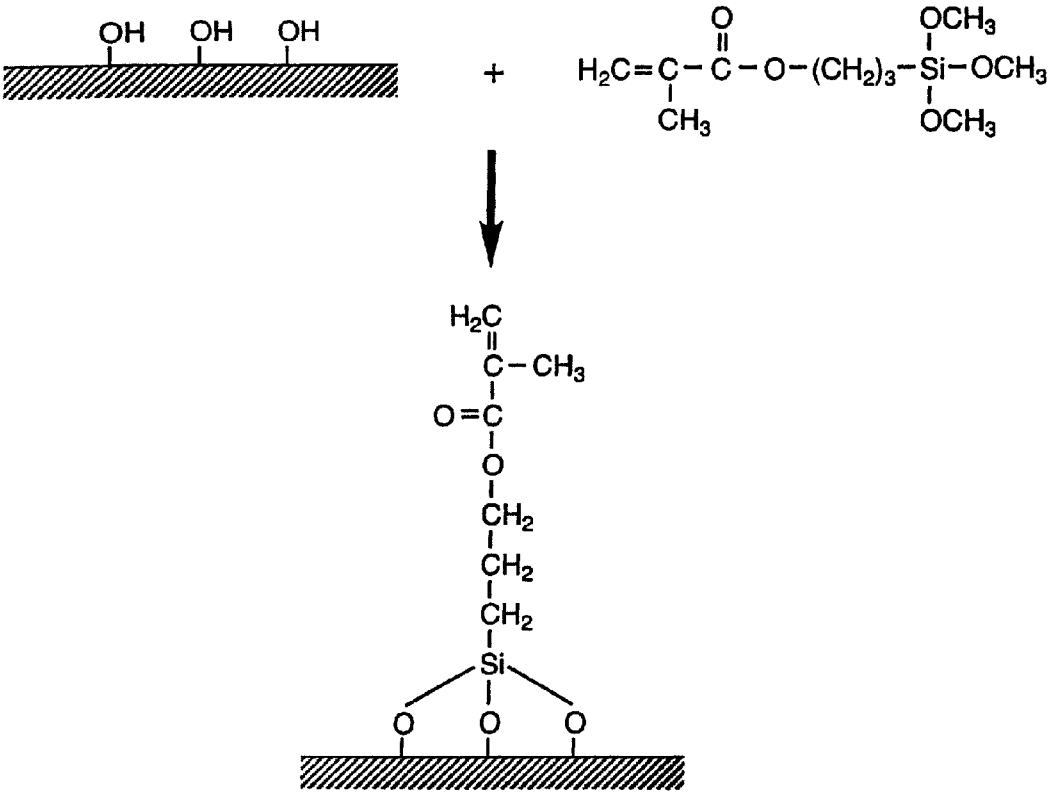

OH
OH
OH
+
O
OCH3
H2C=C-C-O-(CH2)3-Si-OCH3
CH3
OCH3
H2C
C-CH3
O=C
O
CH2
CH2
CH2
Si
O
O
O

FIG. 31

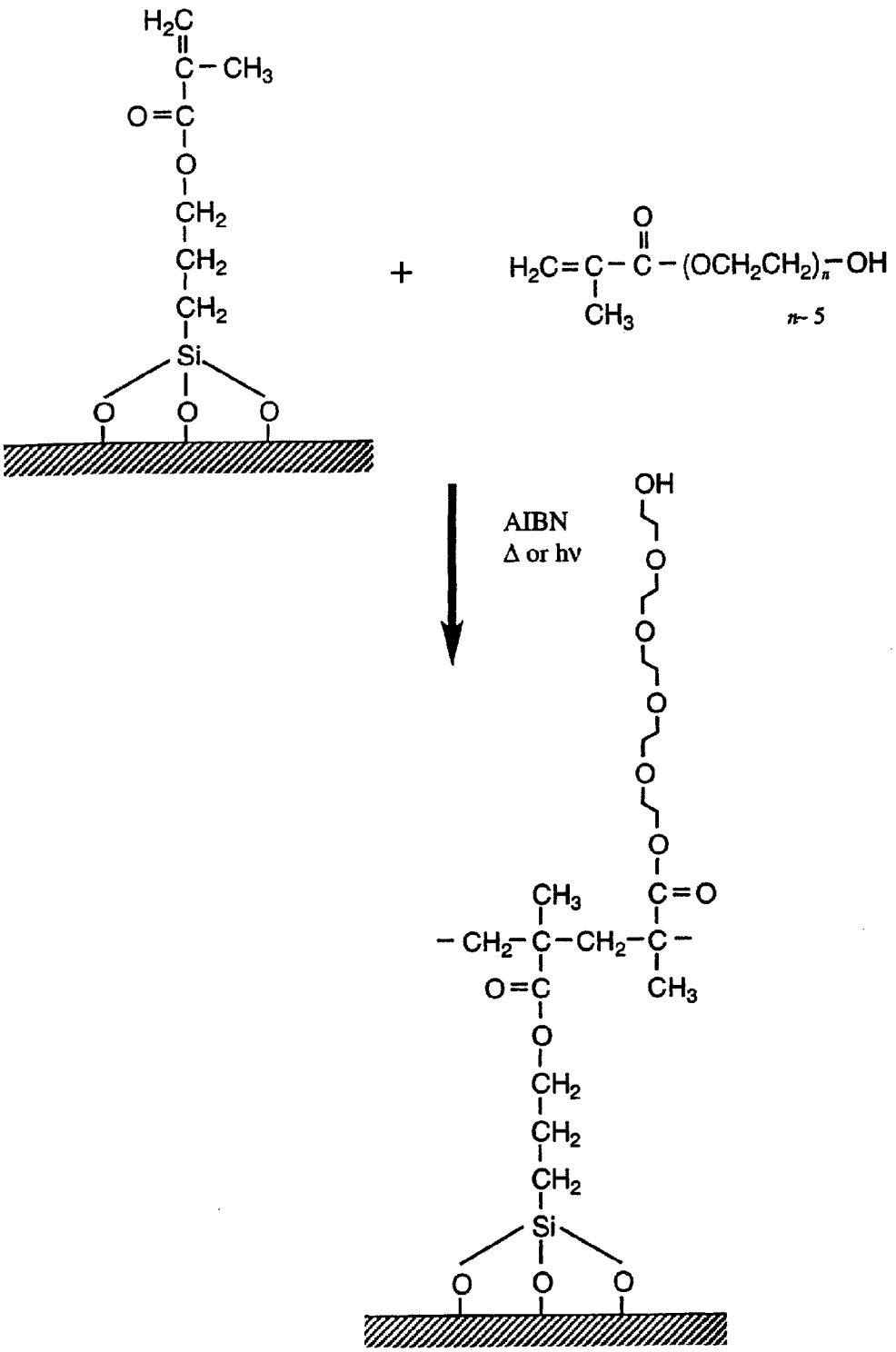


FIG. 32

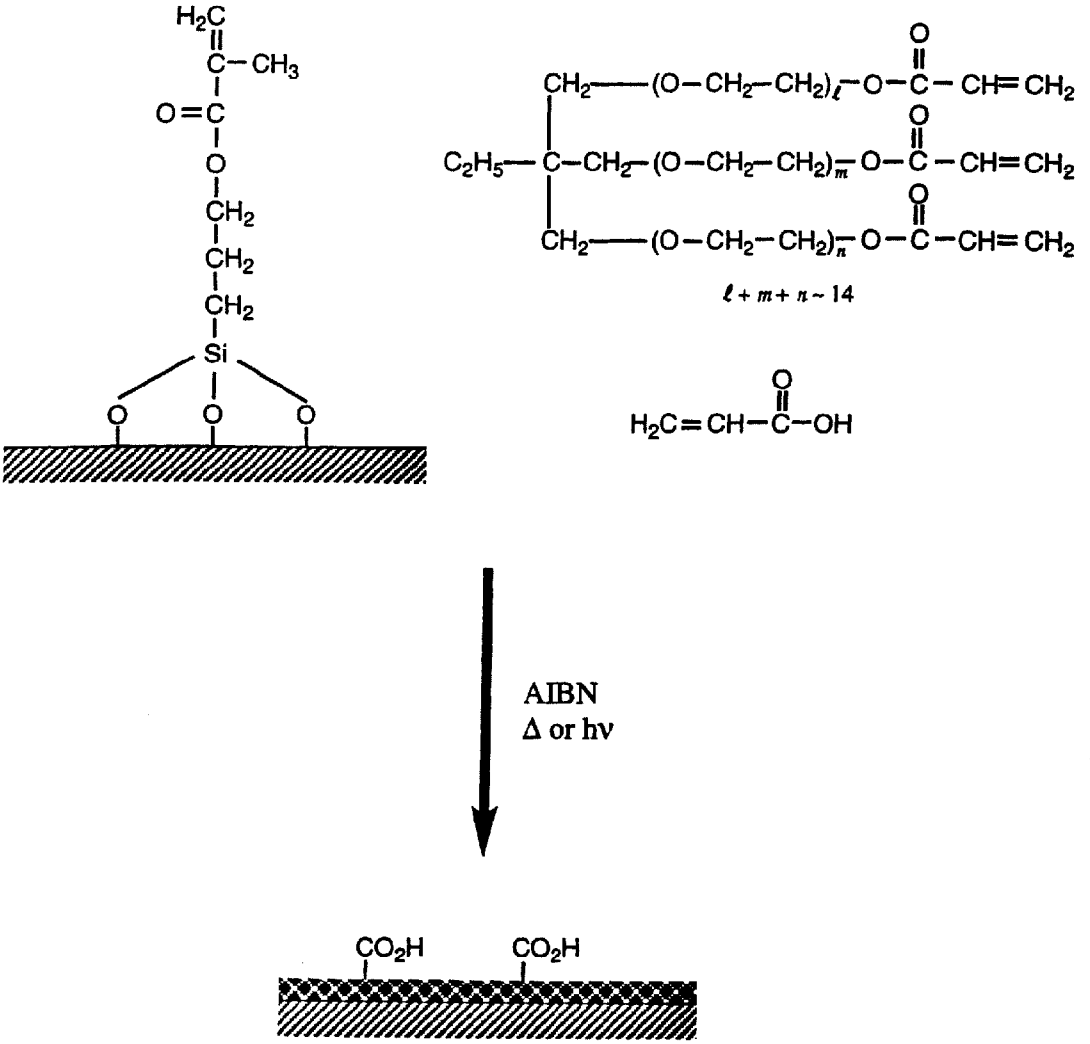


FIG. 33

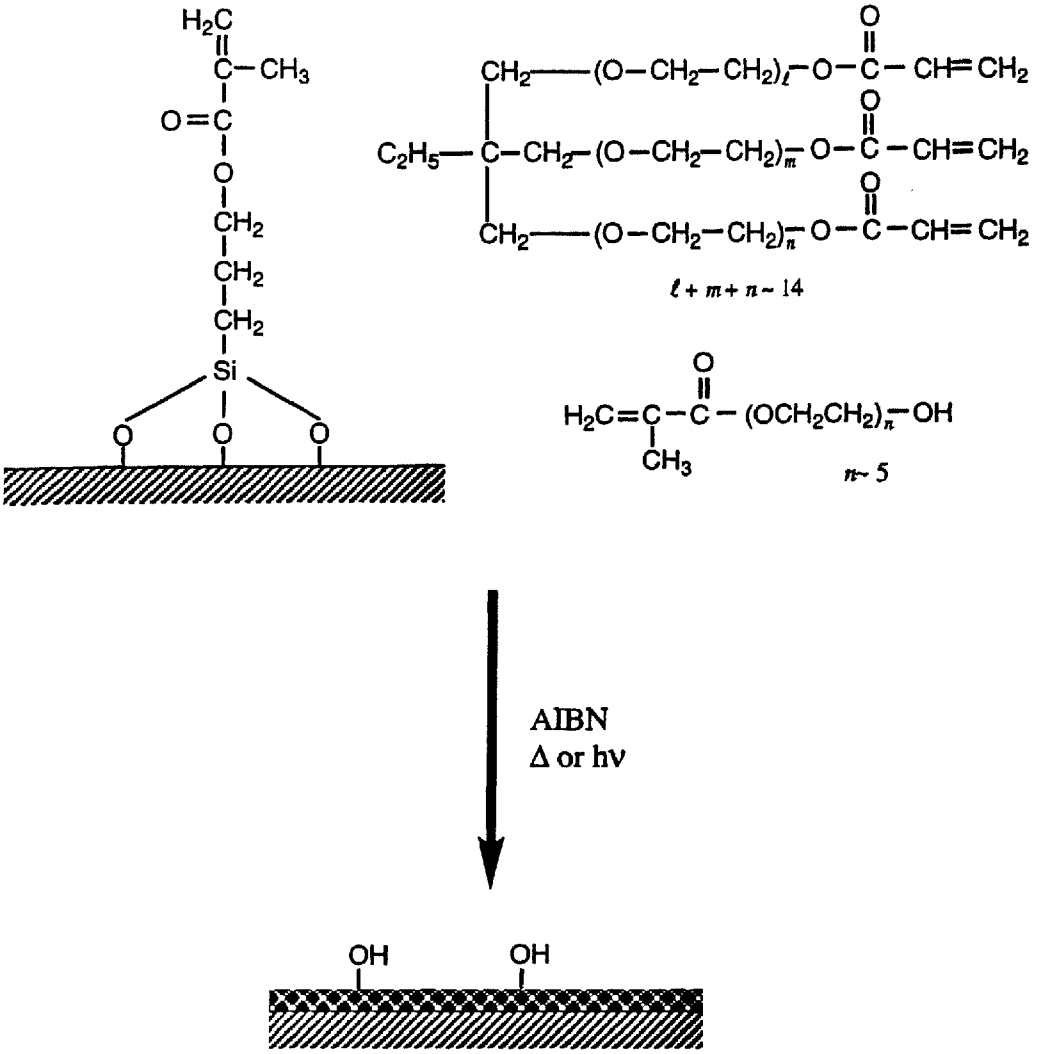


FIG. 34

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

The present application is a continuation of U.S. patent application Ser. No. 10/854,678, filed May 25, 2004, which is a continuation of U.S. patent application Ser. No. 08/794,851, filed Feb. 4, 1997, now U.S. Pat. No. 6,852,487, issued on Feb. 8, 2005, which claims the benefit of U.S. Provisional Patent Application Ser. No. 60/011,359, filed Feb. 9, 1996, which are hereby incorporated by reference in their entirety.

This invention was made with government support under grant number GM-41337-06 awarded by National Institutes of Health. The U.S. Government has certain rights in this invention.

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* 5:86-93 (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 polynucle-

otide. 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 FIGS. **13**A-C, 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**B, 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.

FIG. **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-G.

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-trimethoxy-silane.

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 transloca-

tions 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 4a, 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 4b, 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 2 As 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: 189-93 (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., "Thermophilic 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/Abl, 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, yoghurt, 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 hybridiza-

tion would be in standard antiparallel 5' to 3' and 3' to 5'. Other modified sugar-phosphate backbones would be used in a similar fashion. FIG. **13**A 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). FIGS. **13**B-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.

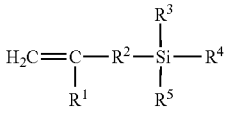
Surfaces on the solid substrate will usually, though not always, be composed of the same material as the substrate. Thus, the surface may be composed of any of a wide variety of materials, for example, polymers, plastics, ceramics, polysaccharides, silica or silica-based materials, carbon, metals, inorganic 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);

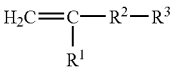
R^3 is an O-alkyl, alkyl, or halogen group;

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 vinyltrimethylsilane.

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, COCl, 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.

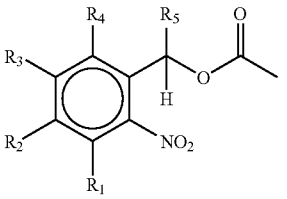
The linker molecules can be attached to the substrate via carbon-carbon bonds using, for example, (poly)tri-fluorochloroethylene surfaces or, preferably, by siloxane bonds (using, for example, glass or silicon oxide surfaces). Siloxane bonds with the surface of the substrate may be formed in one embodiment 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 oligo-tryptophanyl 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,* 13:1008-1017 (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).

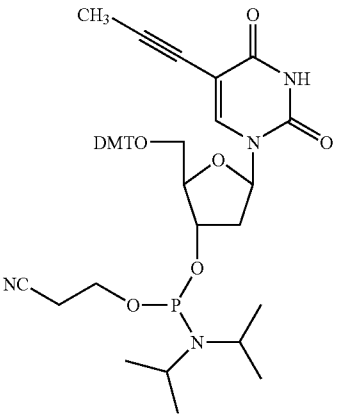
Although considerable progress has been made in constructing oligonucleotide arrays, some containing as many as 256 independent addresses, these procedures are less preferred, for detecting specific DNA sequences by hybridizations. More particularly, arrays containing longer oligonucleotides can currently 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.

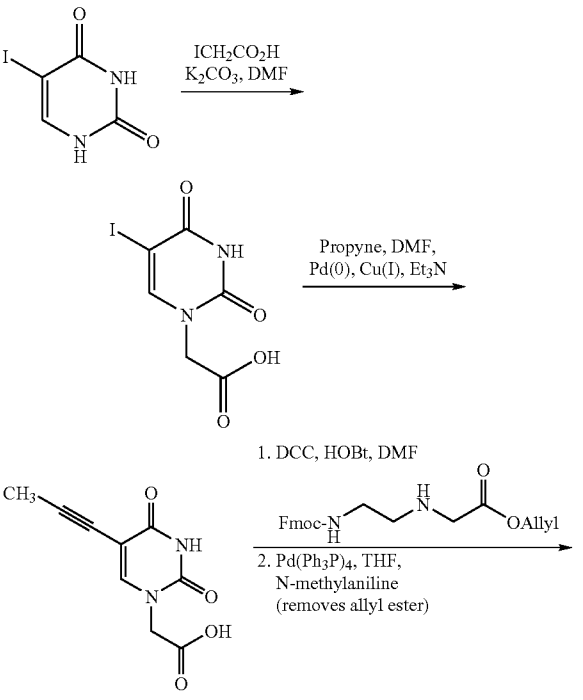
It is desirable to produce a solid support with a good loading of oligonucleotide or PNA oligomer in a relatively small, but well-defined area. Current, commercially available fluorescent imagers can detect a signal as low as 3 attomoles per 50 μm square pixel. Thus, a reasonable size address or "spot" 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.

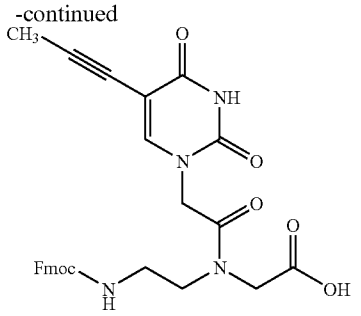
The melting temperature of DNA/DNA duplexes can be estimated as [4n(G·C)+2m(A·T)]° C. Oligonucleotide capture can be optimized by narrowing the T_m difference between duplexes formed by capture oligonucleotides and the complementary addressable array-specific portions hybridized to one another resulting 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):



-continued



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**, I**5**, M**5**, A**9**, E**9**, I**9**, M**9**, A**13**, E**13**, I**13**, 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 se uences.

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 FIG. **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 24-mer 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-G). 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 third round synthesis of PNA rows. The first row contains tetramer 3. FIG. **21**F shows the structure of the array after third round synthesis of columns, adding tetramers 16, 7, 20, 29. Note that all 24-mer 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 at 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 (FIG. **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-**24**B and line **24**C-**24**C 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 (FIG. **25**A-C) will route each tetramer to the appropriate row. Alternatively, a cylindrical manifold (FIG. **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**. FIG. **25**A is a top view of valve block assembly **14**, while side views FIGS. **25**B and **25**C are, respectively, taken along line **25**B-**25**B and line **25**C-**25**C of FIG. **25**A. 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); Jonsson, 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 Enzymol.*, 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 non-specifically 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 25 G ¾ 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 21 G 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 21 G 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 oligonucleotide 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.

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)

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 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			

TABLE 4B-continued

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	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

TABLE 5-continued

Quantification of captured oligonucleotide probes

Crosslinker	Percentage crosslinker	probe 12 (pic)*	probe 12 (amol)
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 mismatches, 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 nmol/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 1.5 ml 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

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	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

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	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

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	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. Jönsson, 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 glove-box 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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What is claimed:

1. A method comprising:

providing an array of a plurality of capture oligonucleotides wherein each type of capture oligonucleotide is greater than 16 nucleotides and differs in nucleotide sequence, when aligned to another type of capture oligonucleotide, by at least 25%, wherein said capture oligonucleotides are coupled to a solid support;

providing a sample comprising a plurality of target oligonucleotides, each target oligonucleotide comprising (i) an addressable array-specific portion, (ii) a further nucleotide sequence, and (iii) a detectable reporter label;

contacting the sample comprising the plurality of target oligonucleotides with the array of capture oligonucleotides under uniform hybridization conditions effective to hybridize the addressable array-specific portion of each target oligonucleotide to its complementary capture oligonucleotide; and

detecting the reporter labels of one or more of the plurality of target oligonucleotides hybridized to their complementary capture oligonucleotides on the solid support.

2. The method according to claim **1**, wherein each capture oligonucleotide comprises 20-25 nucleotides.

3. The method according to claim **1**, wherein the addressable array-specific portions of the target oligonucleotides hybridize to their complementary capture oligonucleotides under highly stringent uniform hybridization conditions.

4. The method according to claim **1**, wherein the addressable array-specific portions of the target oligonucleotides hybridize to their complementary capture oligonucleotides with complete complementarity.

5. A method comprising:

providing a collection of capture oligonucleotides wherein each type of capture oligonucleotide in the collection comprises a nucleotide sequence that is greater than sixteen nucleotides and differs from nucleotide

sequences of other types of capture oligonucleotides in the collection, when aligned to each other, by at least 25%;

providing a sample comprising a plurality of target oligonucleotides, each oligonucleotide comprising (i) an address-specific portion, (ii) one or more further nucleotide sequence portions, and (iii) a detectable reporter label;

contacting the sample comprising a plurality of target oligonucleotides with the collection of capture oligonucleotides under uniform hybridization conditions effective to hybridize the address-specific portion of each target oligonucleotide to its complementary capture oligonucleotide in the collection with minimal non-specific hybridization; and

detecting the reporter labels of one or more of the plurality of target oligonucleotides hybridized to their complementary capture oligonucleotides.

6. The method according to claim **5**, wherein each capture oligonucleotide comprises 20-25 nucleotides.

7. The method according to claim **5**, wherein the address-specific portions of the target oligonucleotides hybridize to their complementary capture oligonucleotides under highly stringent uniform hybridization conditions.

8. The method according to claim **5**, wherein the address-specific portions of the target oligonucleotides hybridize to their complementary capture oligonucleotide with complete complementarity.

9. The method according to claim **5**, wherein said detecting is carried out on a solid support.

10. A method for identifying one or more target nucleotide sequences in a sample, said method comprising:

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

providing a plurality of oligonucleotide probe sets, each set characterized by (a) a first oligonucleotide probe, having a target-specific portion and an addressable array-specific portion and (b) a second oligonucleotide probe, having a target-specific portion;

providing a ligase;

blending the sample, the plurality of oligonucleotide probe sets, and the ligase to form a mixture;

subjecting the mixture to one or more ligase detection reaction cycles to form a ligated product sequence containing (a) the addressable array-specific portion and (b) the target-specific portions, if their respective target nucleotide is present in the sample;

providing a solid support with a plurality of types of capture oligonucleotides immobilized at particular sites, wherein the plurality of types of capture oligonucleotides, each having greater than 16 nucleotides, comprise nucleotide sequences that are complementary to the addressable array-specific portions, and wherein each type of capture oligonucleotide differs in nucleotide sequence, when aligned to each other, by at least 25% to reduce non-specific hybridization between non-complementary capture oligonucleotides and addressable array-specific portions;

contacting the mixture, after said subjecting, with the solid support under uniform hybridization conditions effective to hybridize the addressable array-specific portions to their complementary capture oligonucleotides in a base-specific manner, thereby capturing the addressable array-specific portions on the solid support at the site with the complementary capture oligonucleotide; and

detecting the ligated product sequences captured to the solid support at particular sites, thereby indicating the presence of one or more target nucleotide sequences in the sample.

11. The method according to claim **10**, wherein the first and second oligonucleotide probes hybridize at adjacent positions in a base-specific manner to their respective target nucleotide sequence, if present in the sample, to form a ligation junction and ligate to one another due to perfect complementarity at the ligation junction, but, when the first and second 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 such ligation.

12. The method according to claim **11**, wherein the mismatch is at the 3' base at the ligation junction.

13. The method according to claim **10**, wherein the first and second oligonucleotide probes hybridize at adjacent positions in a base-specific manner to their respective target nucleotide sequence, if present in the sample, to form a ligation junction and ligate to one another due to perfect complementarity at the ligation junction, but, when the first and second oligonucleotide probes in the set are hybridized to any other nucleotide sequence present in the sample, there is a mismatch at a base adjacent to a base at the ligation junction which interferes with such ligation.

14. The method according to claim **13**, wherein the mismatch is at the base adjacent to the 3' base at the ligation junction.

15. The method according to claim **10**, wherein a ligation detection reaction cycle comprises a denaturation treatment, wherein any hybridized oligonucleotide probes are separated from their target nucleotide sequences, and a hybridization treatment, wherein the target-specific oligonucleotide probe portions hybridize in a base-specific manner to their respective target nucleotide sequences, if present in the sample, and ligate to one another.

16. The method according to claim **15**, wherein each cycle, comprising a denaturation treatment and a hybridization treatment, is from about 30 seconds to about five minutes long.

17. The method according to claim **16**, wherein the denaturation treatment is at a temperature of about 80°-105° C.

18. The method according to claim **10**, wherein said subjecting is repeated for 2 to 50 cycles.

19. The method according to claim **10**, wherein total time for said subjecting is 1 to 250 minutes.

20. The method according to claim **10**, wherein the target-specific portions of the oligonucleotide probes each have a hybridization temperature of 20-85° C.

21. The method according to claim **10**, wherein the target-specific portions of the oligonucleotide probes are 20 to 28 nucleotides long.

22. The method according to claim **10** further comprising:

amplifying the target nucleotide sequences in the sample prior to said blending.

23. The method according to claim **22**, wherein said amplifying is carried out by subjecting the sample to a polymerase-based amplifying procedure.

24. The method according to claim **23**, wherein said polymerase-based amplifying procedure is carried out with DNA polymerase.

25. The method according to claim **22**, wherein said amplifying is carried out by subjecting the target nucleotide sequences in the sample to a ligase chain reaction process.

26. The method according to claim **10**, wherein the target-specific portions of the oligonucleotide probe sets are config-

ured to be successfully ligated in the presence of their target sequences under a single set of ligase reaction conditions.

27. The method according to claim **10**, wherein the ligase is selected from the group consisting of *Thermus aquaticus* ligase, *Thermus thermophilus* ligase, *E. coli* ligase, T4 ligase, and *Pyrococcus* ligase.

28. A method for identifying one or more target nucleotide sequences in a sample, said method comprising:

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

providing a plurality of oligonucleotide probe sets, each set characterized by (a) a first oligonucleotide probe, having a target-specific portion and an address-specific portion and (b) a second oligonucleotide probe, having a target-specific portion;

providing a ligase;

blending the sample, the plurality of oligonucleotide probe sets, and the ligase to form a mixture;

subjecting the mixture to one or more ligase detection reaction cycles to form a ligated product sequence containing (a) the address-specific portion and (b) the target-specific portions, if their respective target nucleotide is present in the sample;

providing a collection of capture oligonucleotides wherein each type of capture oligonucleotide in the collection comprises greater than 16 nucleotides and comprises a nucleotide sequence complementary to an address-specific portion, wherein each type of capture oligonucleotide in the collection differs in nucleotide sequence, when aligned to another type of capture oligonucleotide in the collection, by at least 25% of their nucleotides to reduce non-specific hybridization between non-complementary capture oligonucleotides and address-specific portions;

contacting the mixture, after said subjecting, with the collection of capture oligonucleotides under uniform hybridization conditions effective to hybridize the address-specific portion of each ligation product sequence to its capture oligonucleotide comprising a complementary nucleotide sequence in a base-specific manner; and

detecting the ligated product sequences hybridized to their complementary capture oligonucleotides, thereby indicating the presence of one or more target nucleotide sequences in the sample.

29. The method according to claim **28**, wherein the first and second oligonucleotide probes hybridize at adjacent positions in a base-specific manner to their respective target nucleotide sequence, if present in the sample, to form a ligation junction and ligate to one another due to perfect complementarity at the ligation junction, but, when the first and second 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 such ligation.

30. The method according to claim **29**, wherein the mismatch is at the 3' base at the ligation junction.

31. The method according to claim **28**, wherein the first and second oligonucleotide probes hybridize at adjacent positions in a base-specific manner to their respective target nucleotide sequence, if present in the sample, to form a ligation junction and ligate to one another due to perfect complementarity at the ligation junction, but, when the first and second oligonucleotide probes in the set are hybridized to any other nucleotide sequence present in the sample, there is a mismatch at a base adjacent to a base at the ligation junction which interferes with such ligation.

32. The method according to claim **31**, wherein the mismatch is at the base adjacent to the 3' base at the ligation junction.

33. The method according to claim **28**, wherein a ligation detection reaction cycle comprises a denaturation treatment, wherein any hybridized oligonucleotide probes are separated from their target nucleotide sequences, and a hybridization treatment, wherein the target-specific oligonucleotide probe portions hybridize in a base-specific manner to their respective target nucleotide sequences, if present in the sample, and ligate to one another.

34. The method according to claim **33**, wherein each cycle, comprising a denaturation treatment and a hybridization treatment, is from about 30 seconds to about five minutes long.

35. The method according to claim **34**, wherein the denaturation treatment is at a temperature of about 80°-105° C.

36. The method according to claim **28**, wherein said subjecting is repeated for 2 to 50 cycles.

37. The method according to claim **28**, wherein total time for said subjecting is 1 to 250 minutes.

38. The method according to claim **28**, wherein the target-specific portions of the oligonucleotide probes each have a hybridization temperature of 20-85° C.

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

40. The method according to claim **28** further comprising:

amplifying the target nucleotide sequences in the sample prior to said blending.

41. The method according to claim **40**, wherein said amplifying is carried out by subjecting the sample to a polymerase-based amplifying procedure.

42. The method according to claim **41**, wherein said polymerase-based amplifying procedure is carried out with DNA polymerase.

43. The method according to claim **40**, wherein said amplifying is carried out by subjecting the target nucleotide sequences in the sample to a ligase chain reaction process.

44. The method according to claim **28**, wherein the target-specific portions of the oligonucleotide probe sets are configured to be successfully ligated in the presence of their target sequences under a single set of ligase reaction conditions.

45. The method according to claim **28**, wherein the ligase is selected from the group consisting of *Thermus aquaticus* ligase, *Thermus thermophilus* ligase, *E. coli* ligase, T4 ligase, and *Pyrococcus* ligase.

46. The method according to claim **28**, wherein said detecting is carried out on a solid support.

* * * * *

Exhibit J

US007892747B2

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

(10) **Patent No.: US 7,892,747 B2**
(45) **Date of Patent: Feb. 22, 2011**

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

(75) Inventors: **Francis Barany**, New York, NY (US); **George Barany**, Falcon Heights, MN (US); **Robert P. Hammer**, Baton Rouge, LA (US); **Maria Kempe**, Malmo (SE); **Herman Blok**, Wemeldinge (NL); **Monib Zirvi**, Monmouth Junction, NJ (US)

(73) Assignee: **Cornell Research Foundation, Inc.**, Ithaca, NY (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.: **12/725,248**

(22) Filed: **Mar. 16, 2010**

(65) **Prior Publication Data**

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Related U.S. Application Data

(63) Continuation of application No. 10/854,678, filed on May 25, 2004, which is a continuation of application No. 08/794,851, filed on Feb. 4, 1997, now Pat. No. 6,852,487.

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

(51) **Int. Cl.**
C12Q 1/68 (2006.01)

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

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

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(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.

26 Claims, 34 Drawing Sheets

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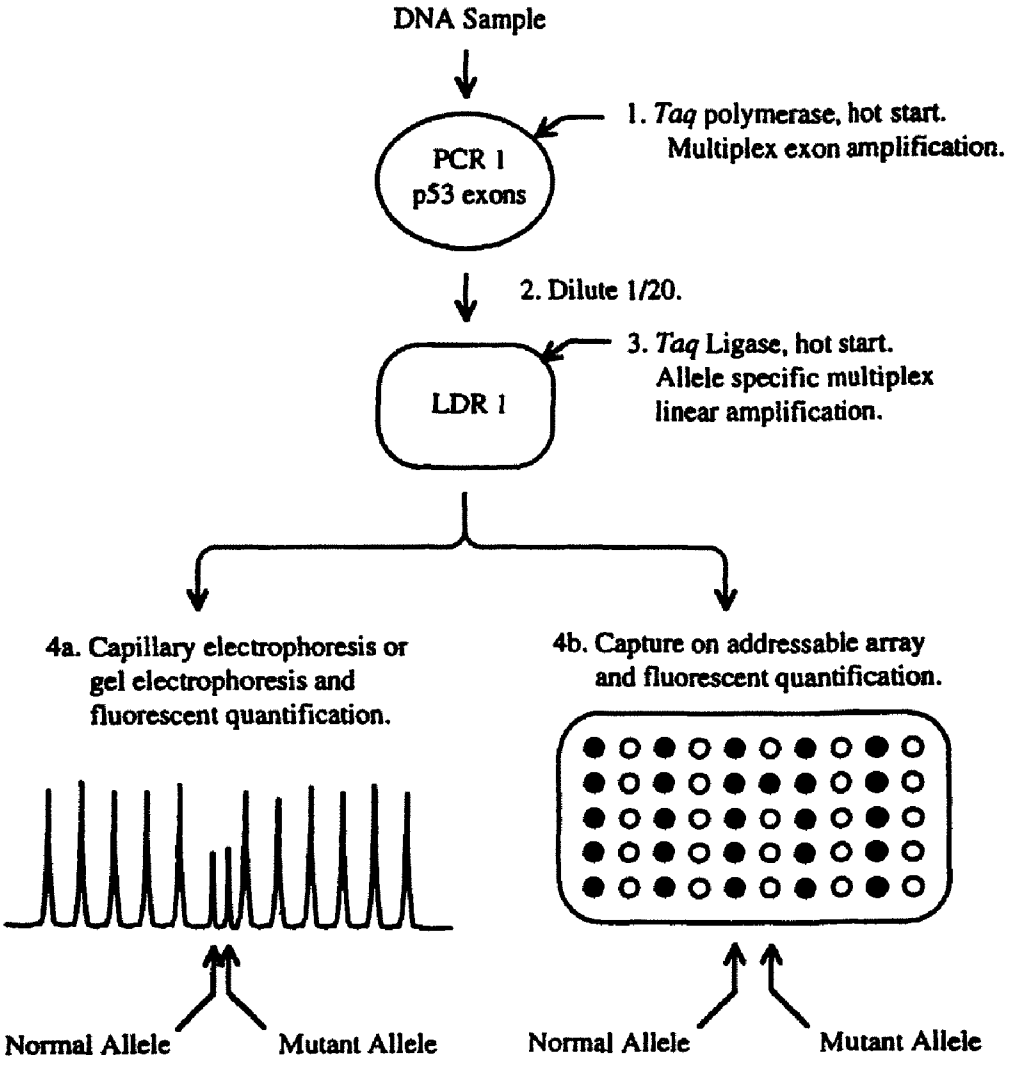

DNA Sample
1. *Taq* polymerase, hot start. Multiplex exon amplification.
PCR 1 p53 exons
2. Dilute 1/20.
3. *Taq* Ligase, hot start. Allele specific multiplex linear amplification.
LDR 1
4a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
4b. Capture on addressable array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

FIG. 1

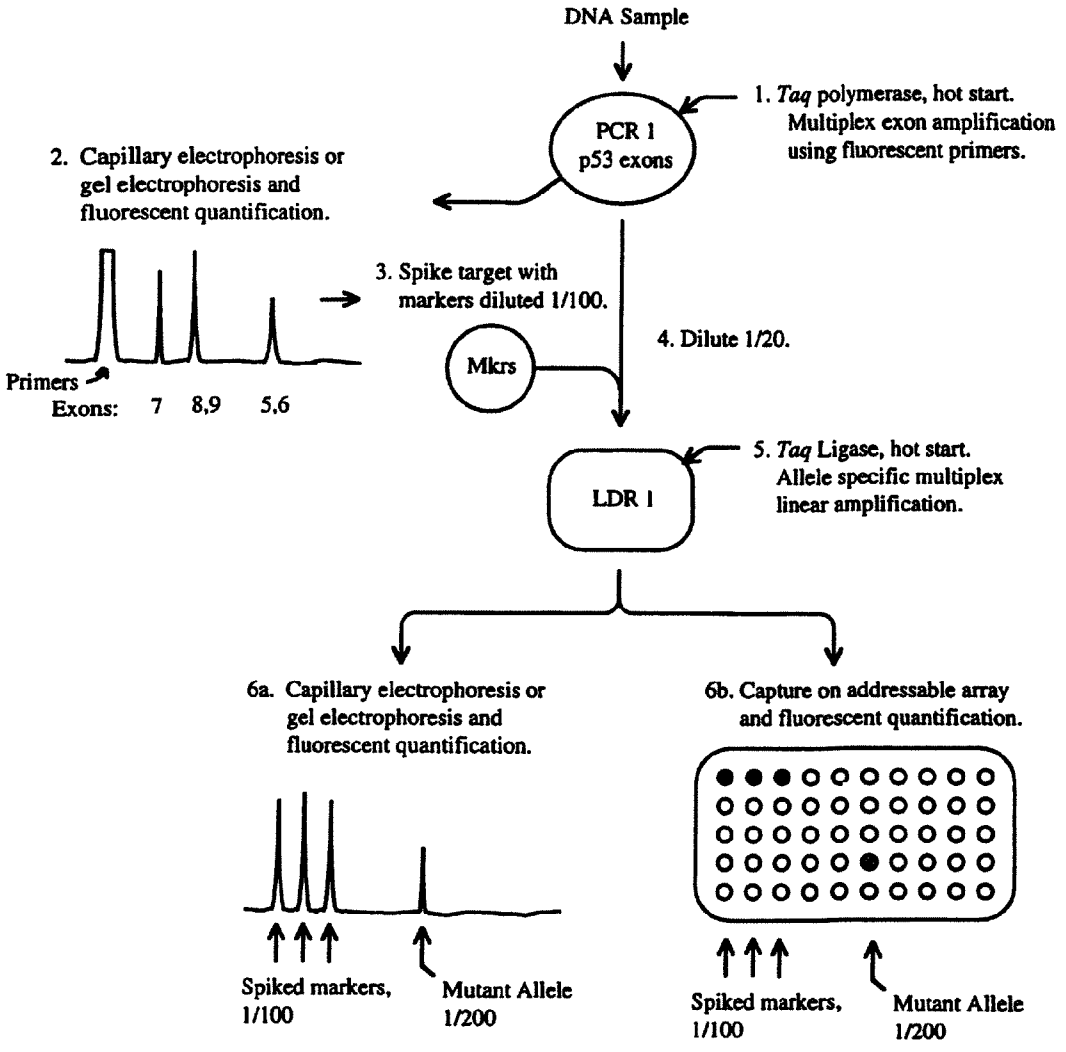

DNA Sample
1. Taq polymerase, hot start. Multiplex exon amplification using fluorescent primers.
PCR 1 p53 exons
2. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
3. Spike target with markers diluted 1/100.
Mkrs
4. Dilute 1/20.
Primers
Exons: 7 8,9 5,6
LDR 1
5. Taq Ligase,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

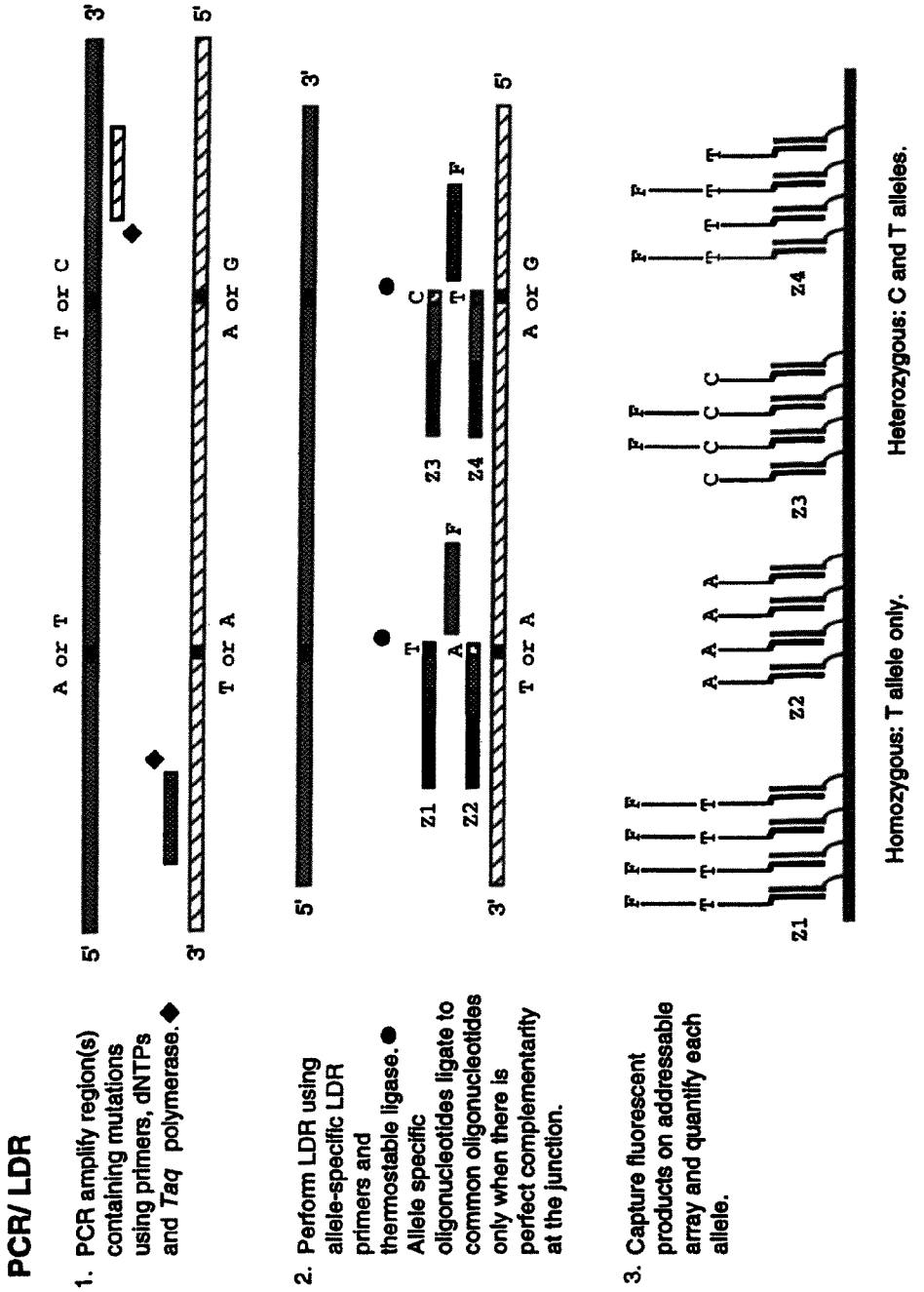

PCR/ LDR
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
3.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

FIG. 3

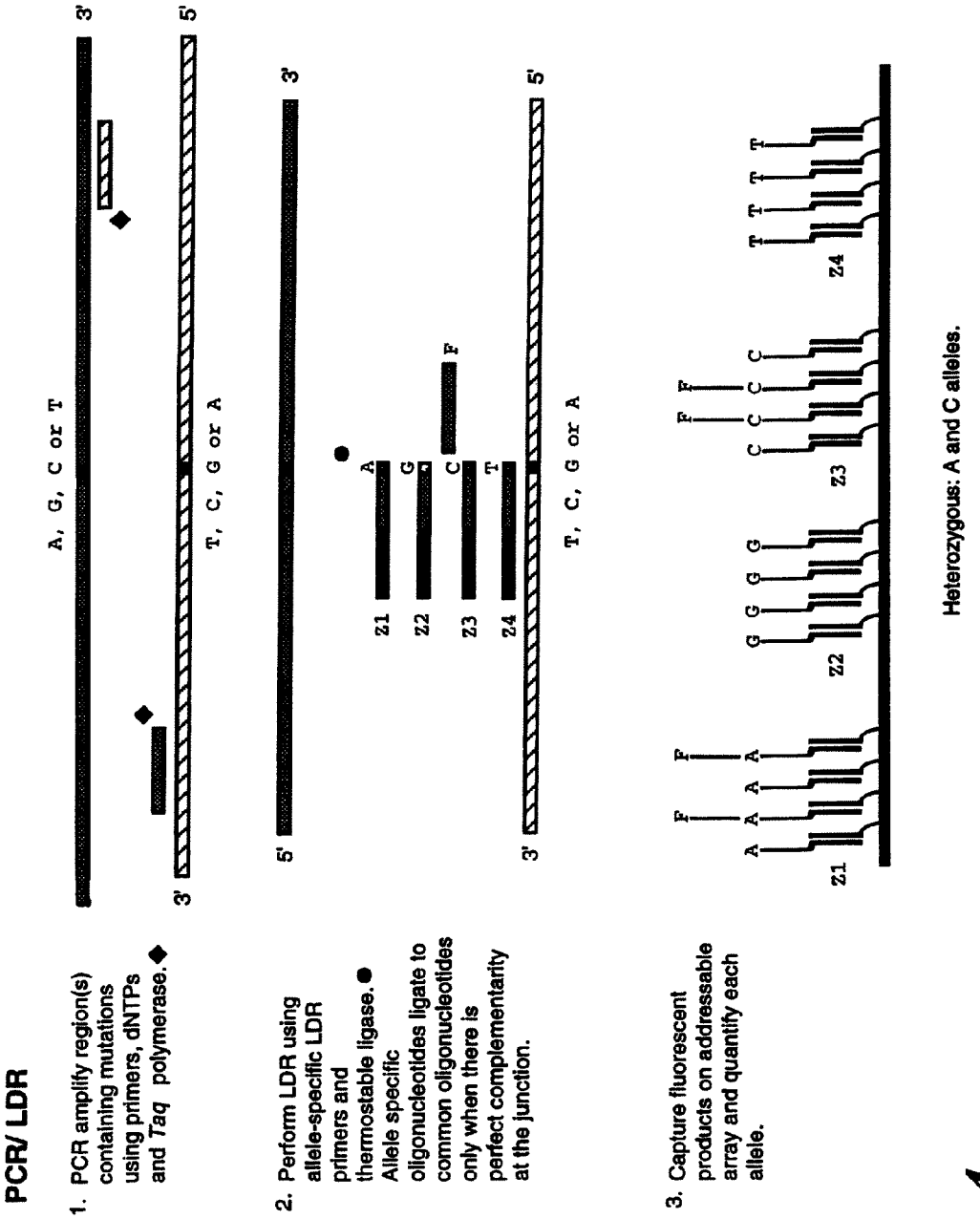

PCR/ LDR
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
3.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

FIG. 4

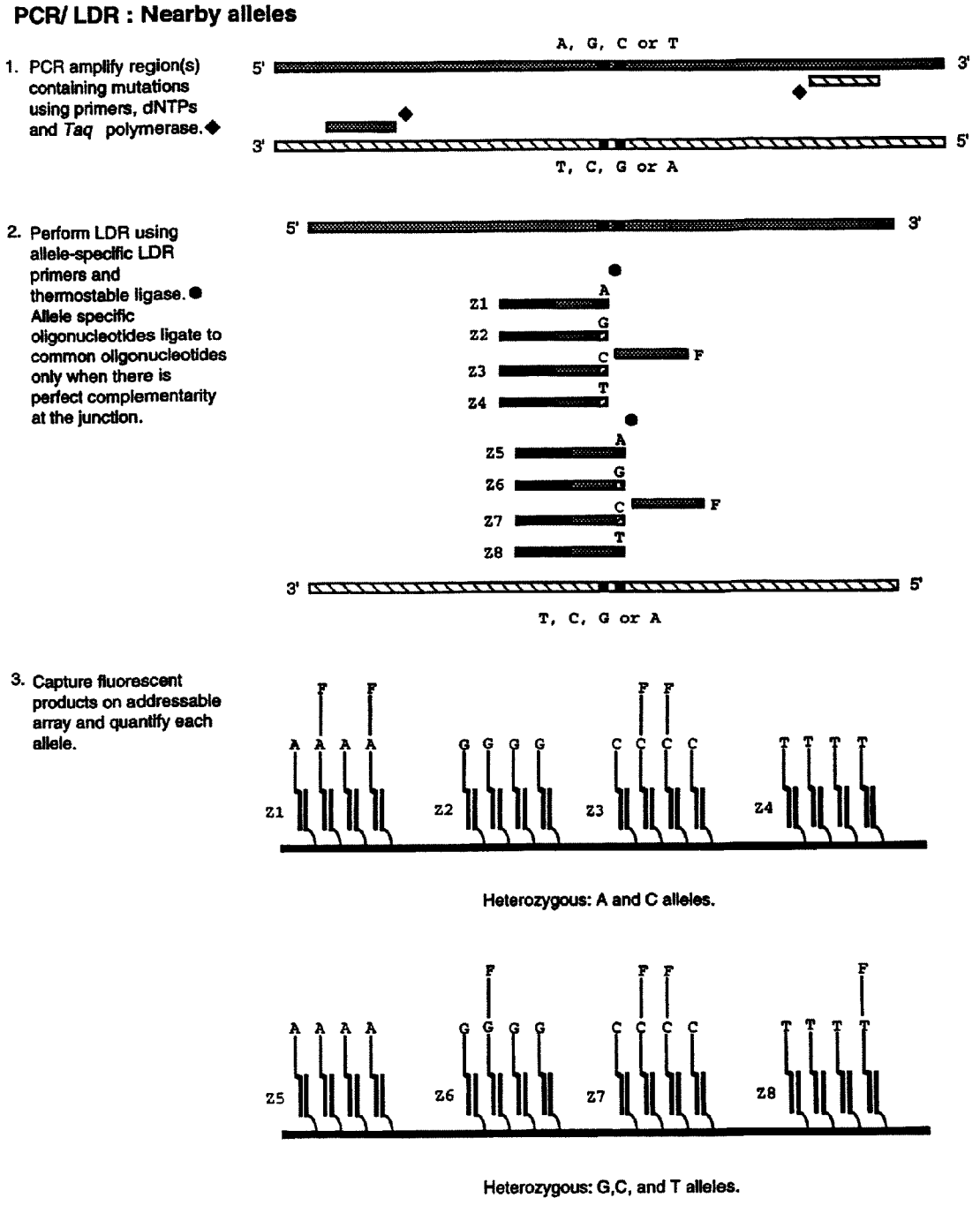

PCR/ LDR : Nearby alleles
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
A, G, C or T
5'
3'
3'
5'
T, C, G or A
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common oligonucleotides only when 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

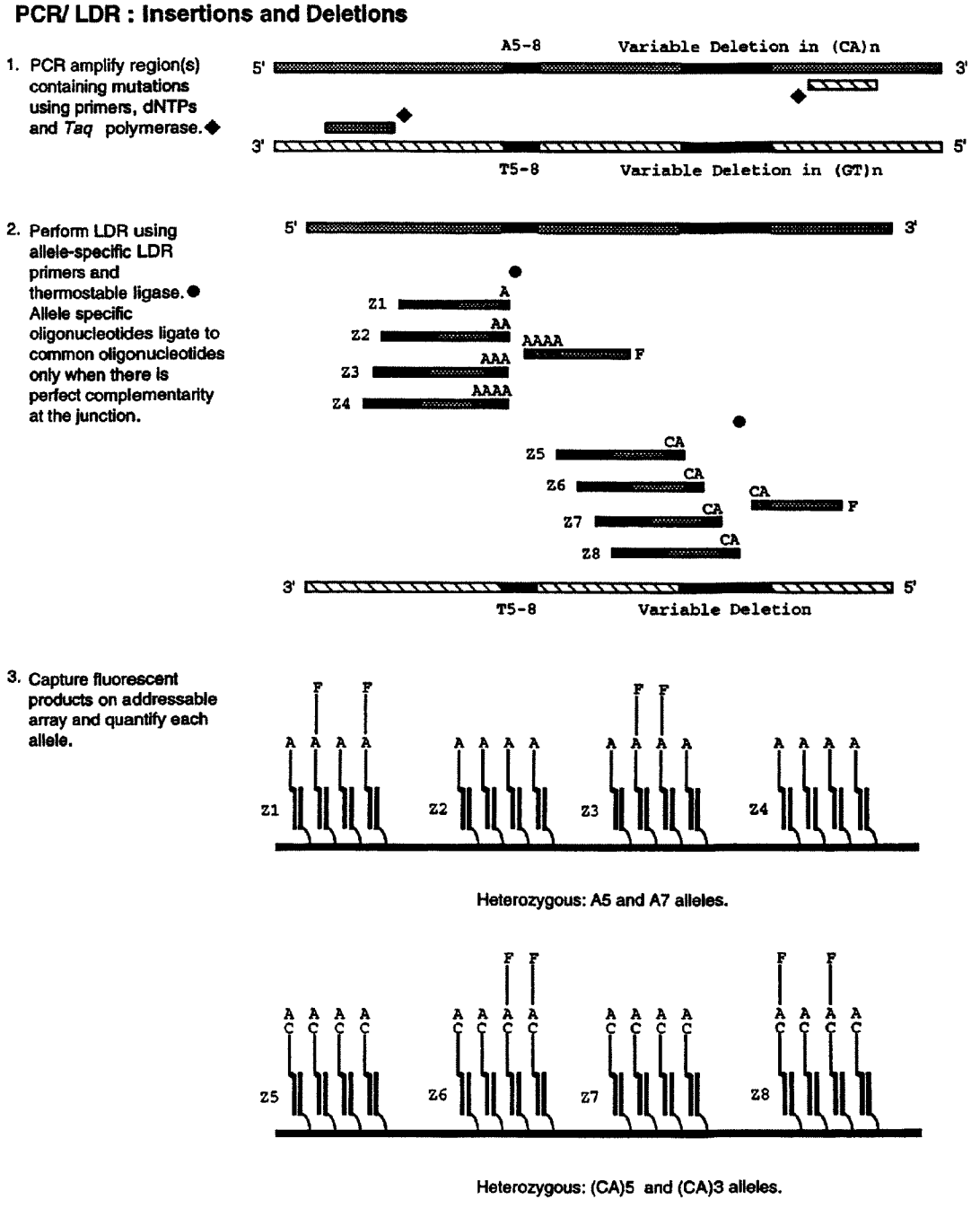

PCR/ LDR : Insertions and Deletions
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
A5-8
Variable Deletion in (CA)n
5'
3'
T5-8
Variable Deletion in (GT)n
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides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

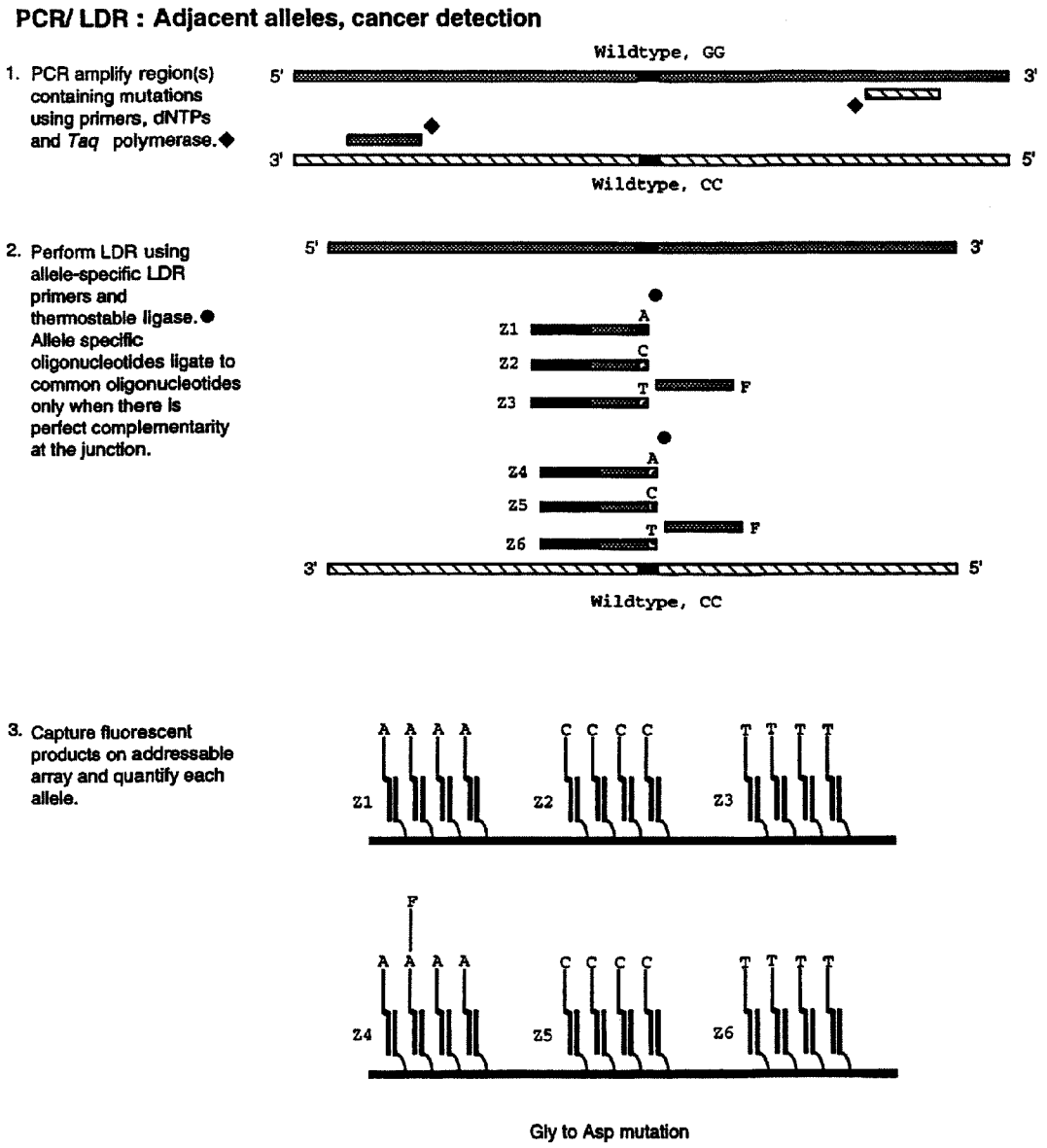

PCR/ LDR : Adjacent alleles, cancer detection
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
Wildtype, GG
5'
3'
3'
5'
Wildtype, CC
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common oligonucleotides 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.
A A A A
C C C C
T T T T
Z1
Z2
Z3
F
A A A A
C C C C
T T T T
Z4
Z5
Z6
Gly to Asp mutation

FIG. 7

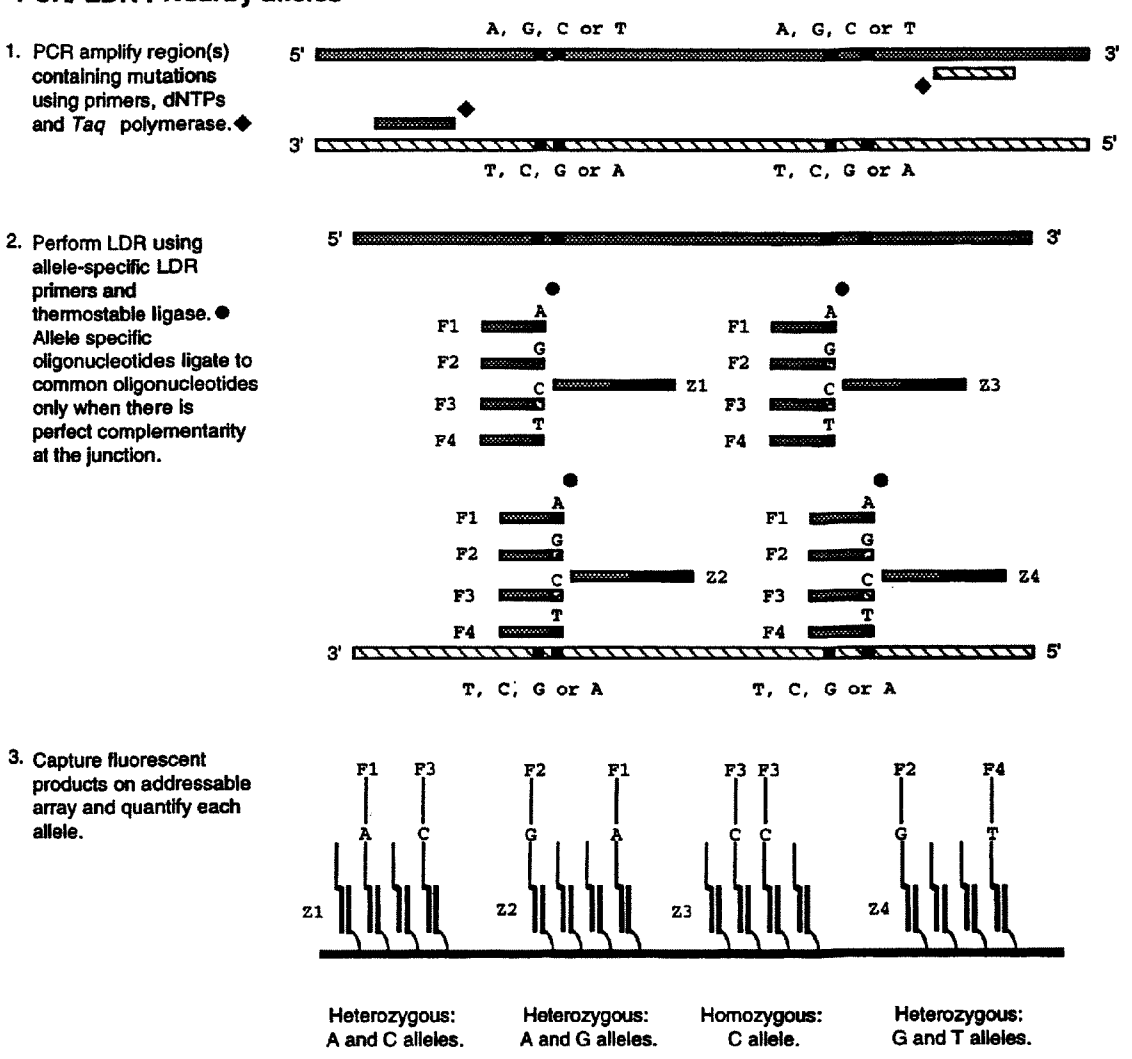

PCR/ LDR : Nearby alleles
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
A, G, C or T
A, G, C or T
5'
3'
3'
5'
T, C, G or A
T, C, G or A
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common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

FIG. 8

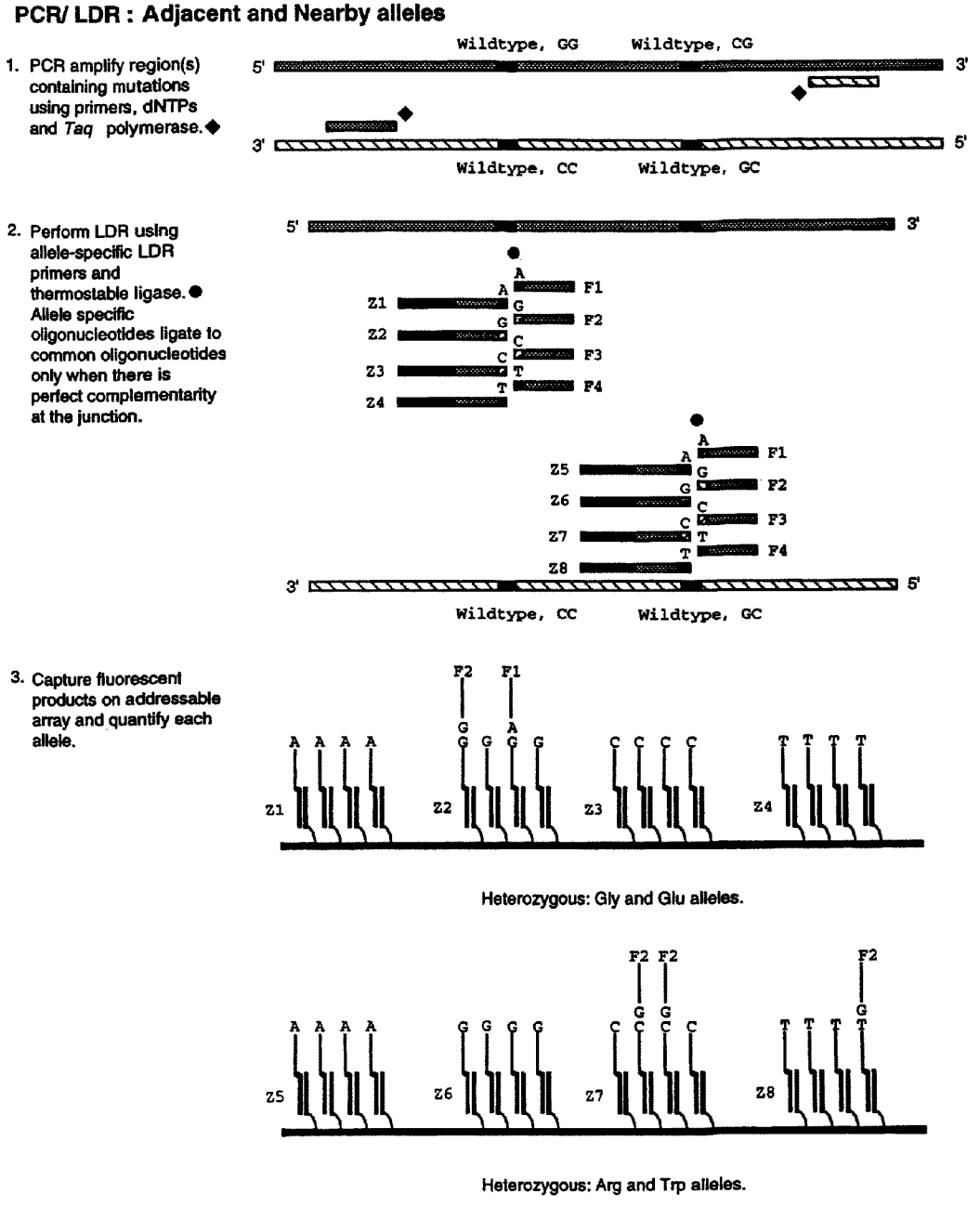

PCR/ LDR : Adjacent and Nearby alleles
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
Wildtype, GG
Wildtype, CG
Wildtype, CC
Wildtype, GC
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate to common 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

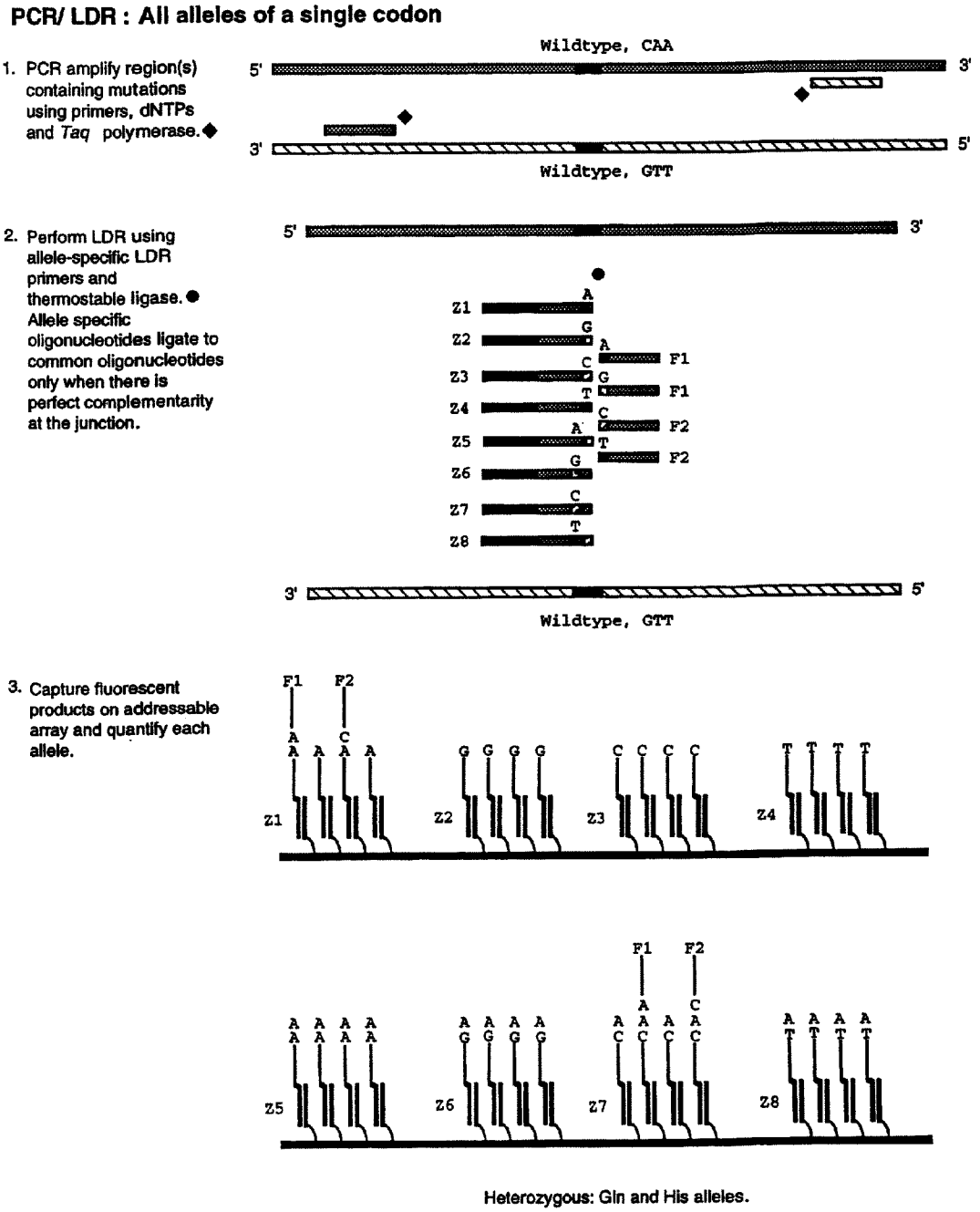

PCR/ LDR : All alleles of a single codon
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
Wildtype, CAA
5'
3'
3'
5'
Wildtype, GTT
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
Z1
Z2
Z3
Z4
Z5
Z6
Z7
Z8
F1
F2
Wildtype, GTT
3. Capture fluorescent products on addressable array and quantify each allele.
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

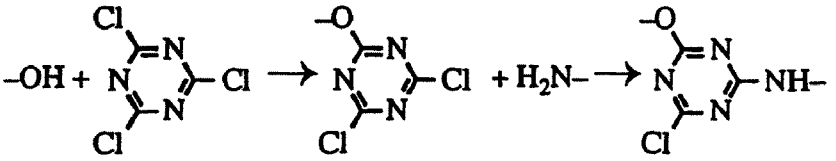
W = protecting group, e.g. Boc, Fmoc
Z = activating group, e.g. imidazole (Im), *p*-nitrophenol (OPnp), hydroxysuccinimide (OSu), pentafluorophenol (OPfp)
PEG = oligo or poly(ethylene glycol), backbone $(CH_2CH_2O)_n$ n = 6 to 200 (can also be grown by anionic polymerization with )
WSC = water soluble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

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



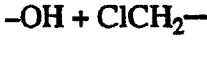
$–OH + O{=}C{=}N– \longrightarrow –O(C{=}O)NH–$
$–O(C{=}S)SCH_2(C{=}O)NH_2 + H_2N– \longrightarrow –O(C{=}S)NH–$
–OH +  + HO– ⟶ $–OCH_2CH(OH)CH_2O–$
(+ H_2NH–) (NH–)
$–OH \longrightarrow –OCH_2(C{=}O)H + H_2N– + NaCNBH_3 \longrightarrow –OCH_2CH_2NH–$

FIG. 11

FIG. 12A

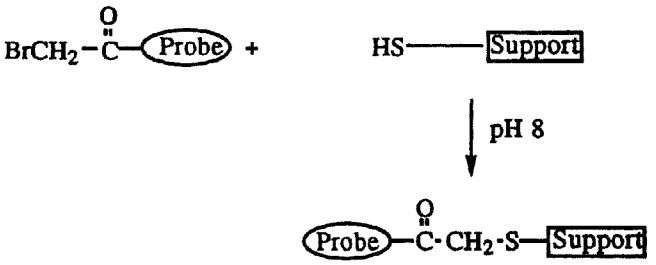


FIG. 12B

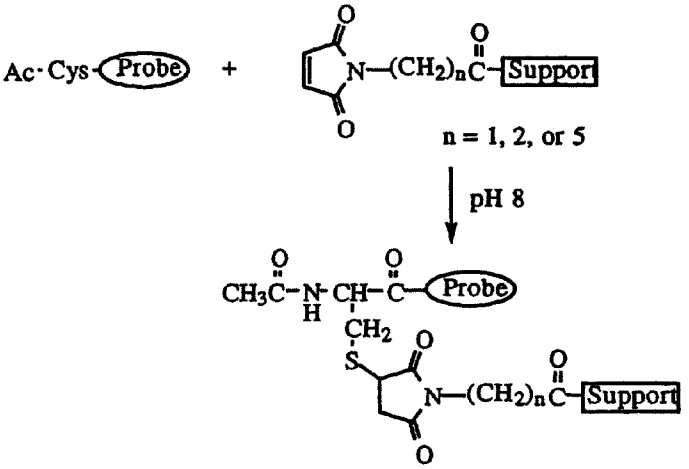
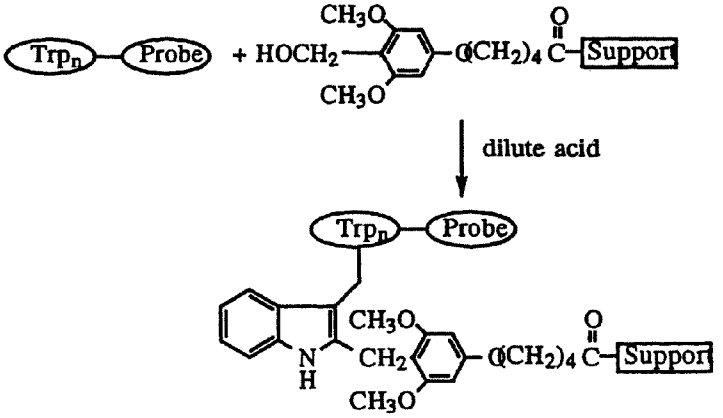


FIG. 12C



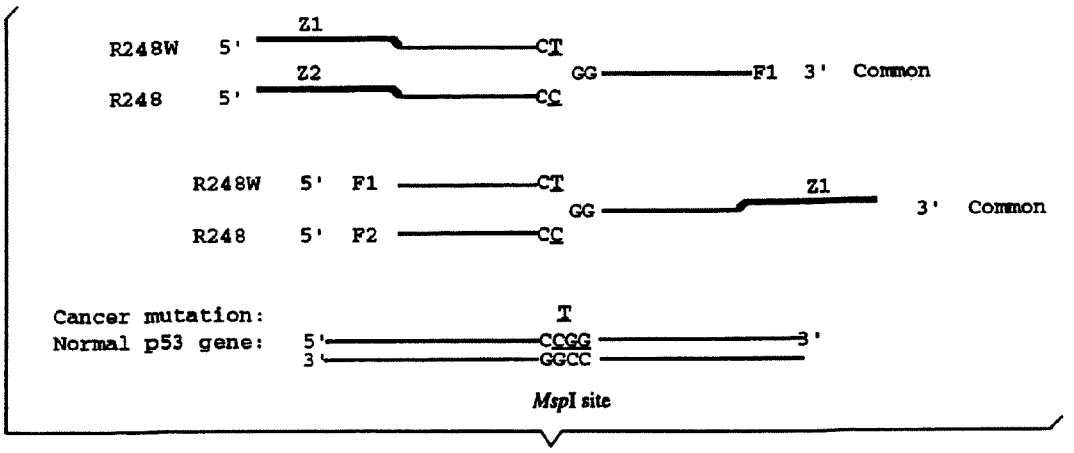


FIG. 13A

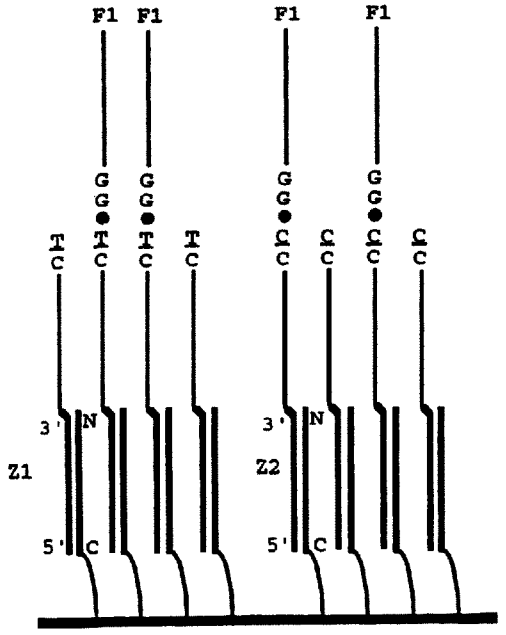


FIG. 13B

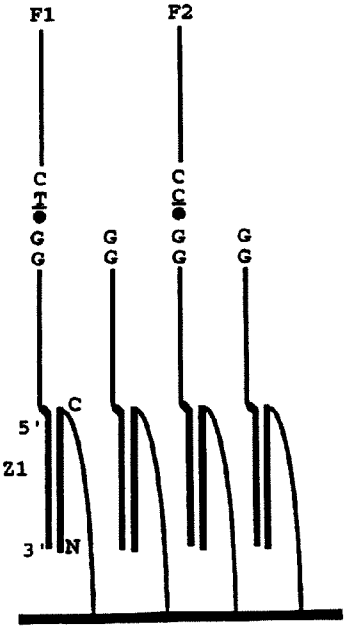


FIG. 13C

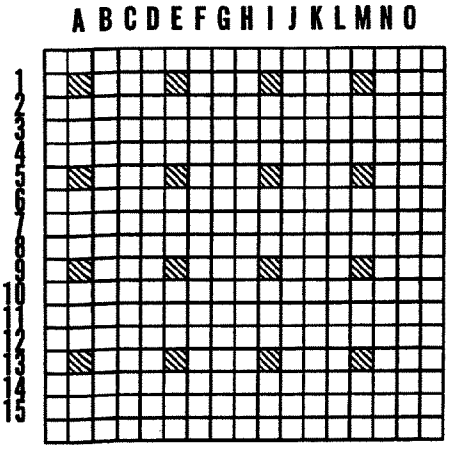


FIG. 14A

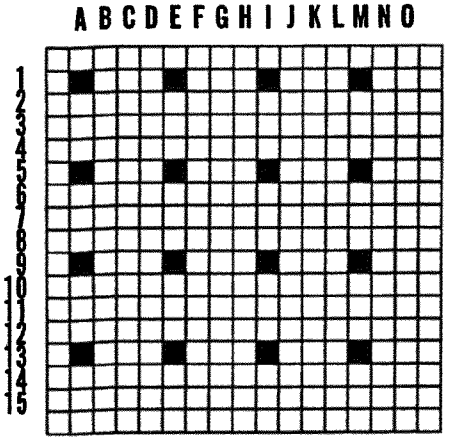


FIG. 14B

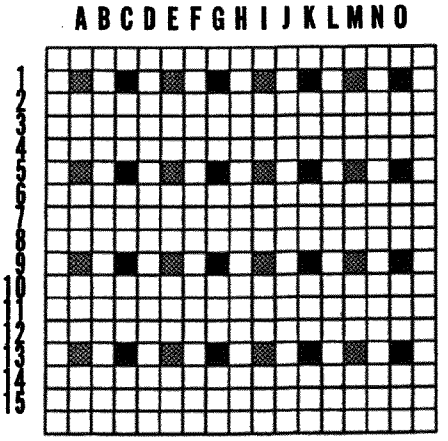


FIG. 14C

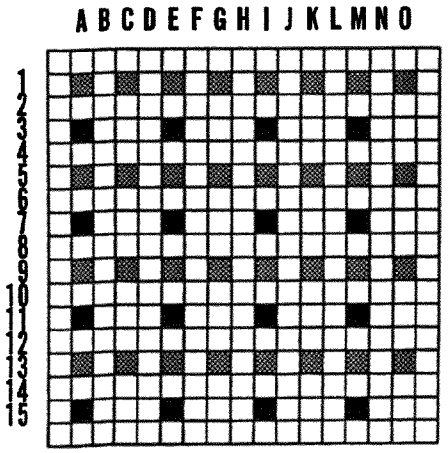


FIG. 14D

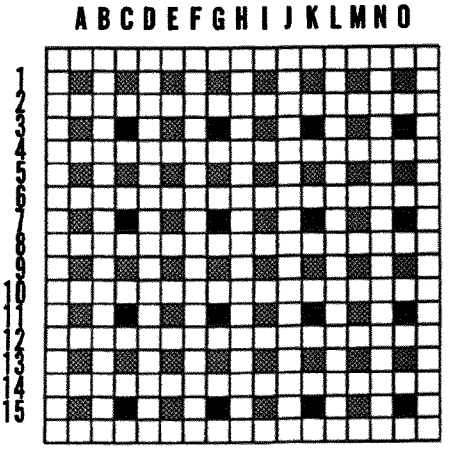


FIG. 14E

FIG. 15A

1st addition of unique 24mers.

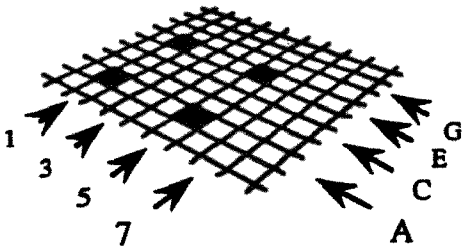


FIG. 15B

2nd addition of unique 24mers.

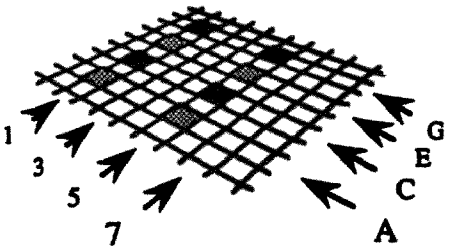


FIG. 15C

3rd addition of unique 24mers.

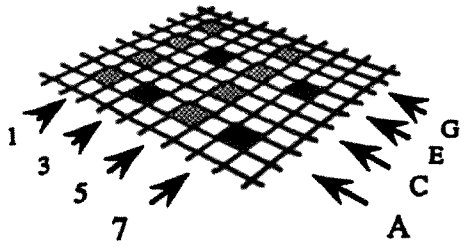
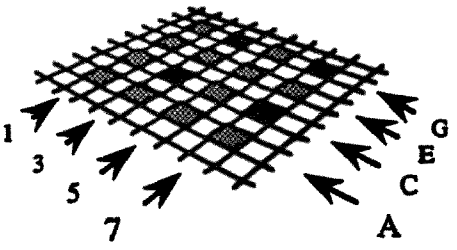


FIG. 15D

4th addition of unique 24mers.



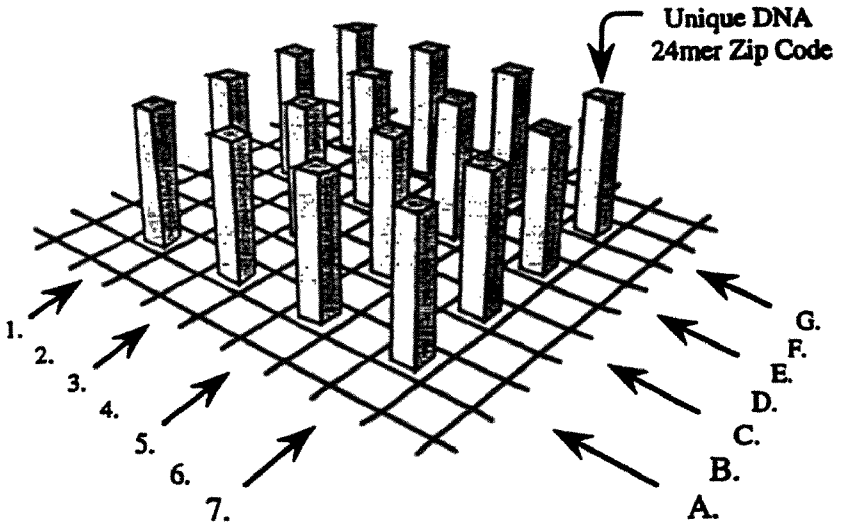


FIG. 15E

FIG. 16A

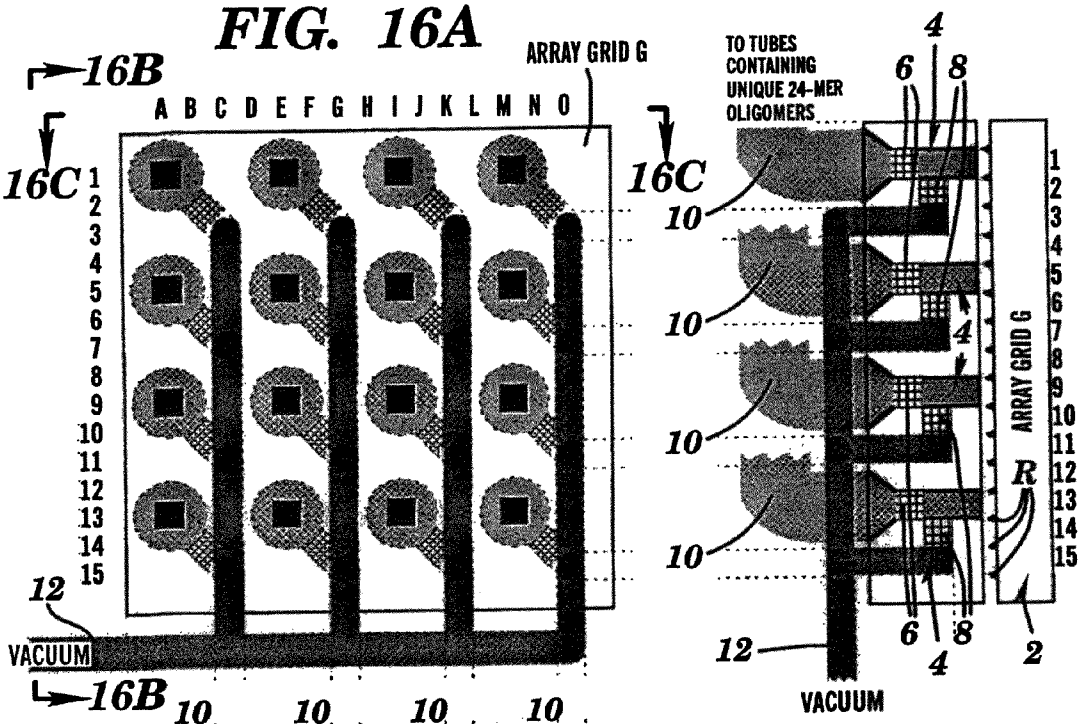


FIG. 16B

FIG. 16C

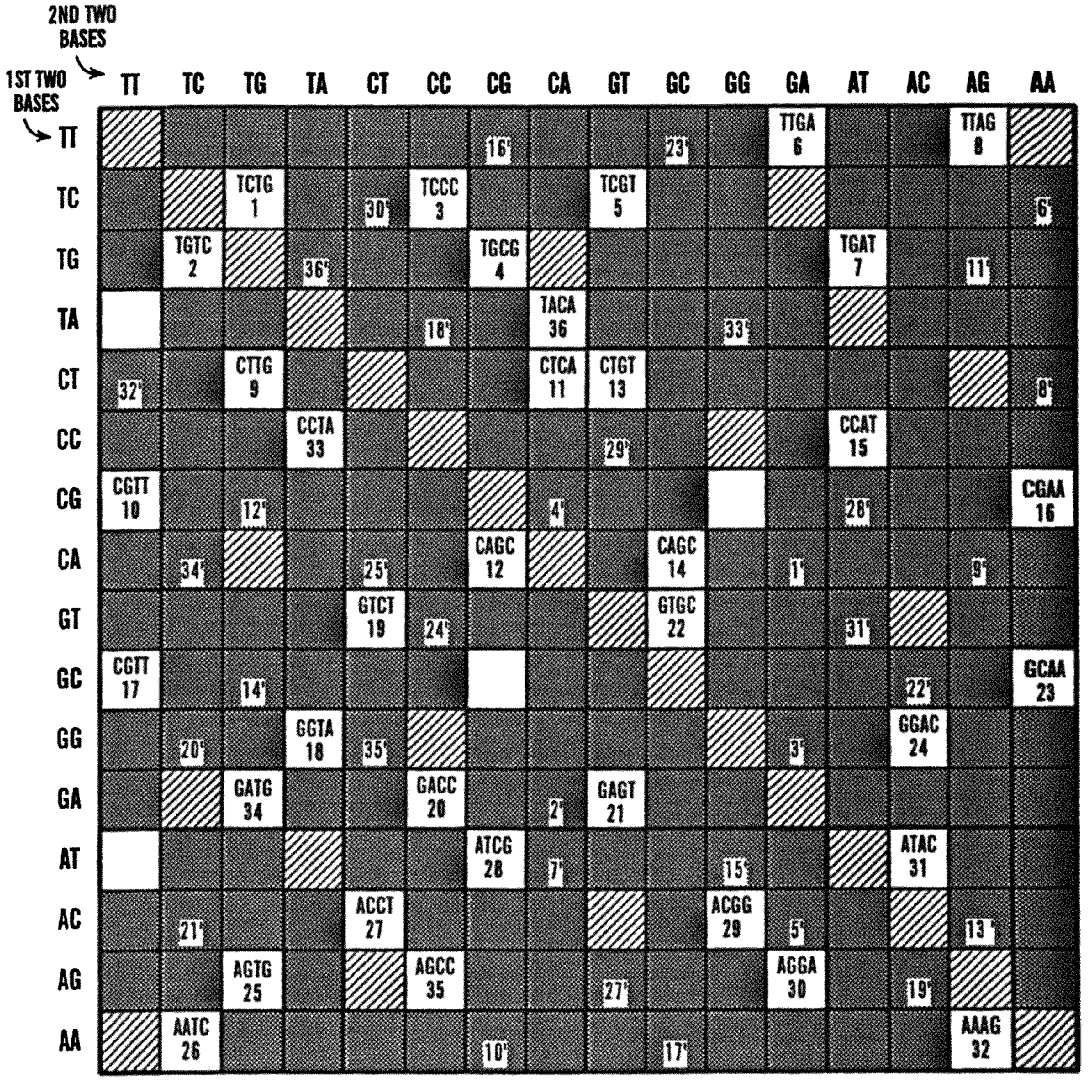

2ND TWO BASES
1ST TWO BASES
TT TC TG TA CT CC CG CA GT GC GG GA AT AC AG AA
TT
TC
TG
TA
CT
CC
CG
CA
GT
GC
GG
GA
AT
AC
AG
AA
TTGA 6
TTAG 8
TCTG 1
TCCC 3
TCGT 5
TGTC 2
TGCG 4
TGAT 7
TACA 36
CTTG 9
CTCA 11
CTGT 13
CCTA 33
CCAT 15
CGTT 10
CGAA 16
CAGC 12
CAGC 14
GTCT 19
GTGC 22
CGTT 17
GCAA 23
GGTA 18
GGAC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

FIG. 17

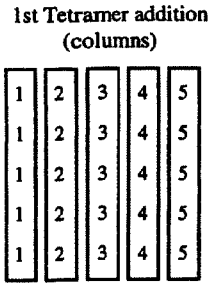


FIG. 18A

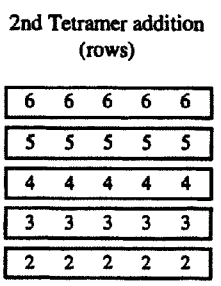


FIG. 18B

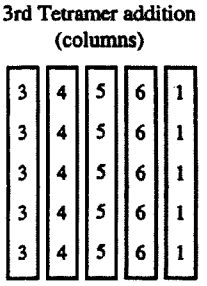


FIG. 18C

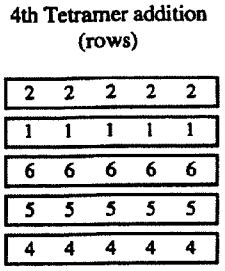


FIG. 18D

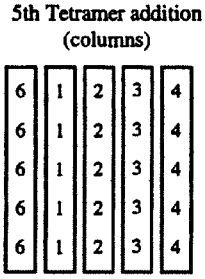


FIG. 18E

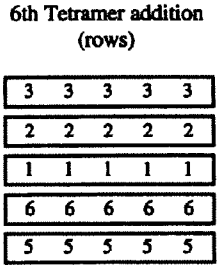


FIG. 18F

Addressable array with full length PNA 24mers

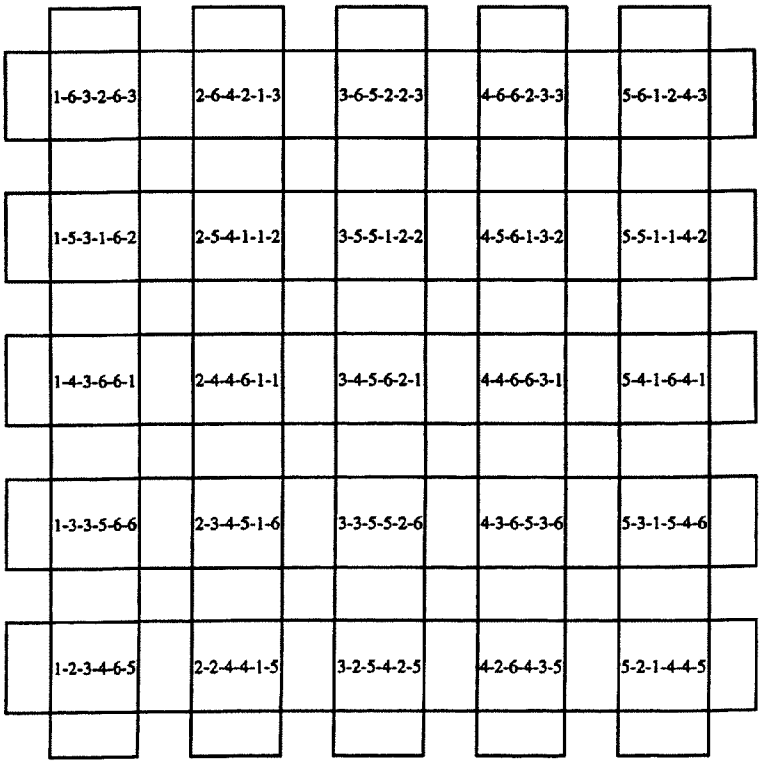


FIG. 18G

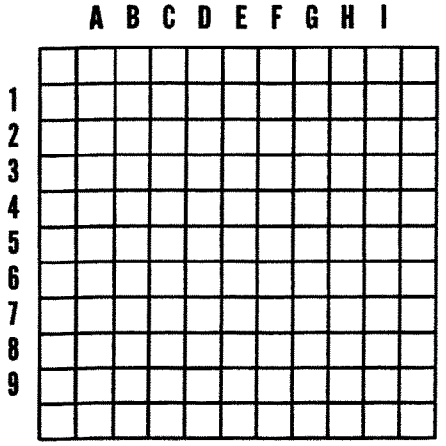


FIG. 19A

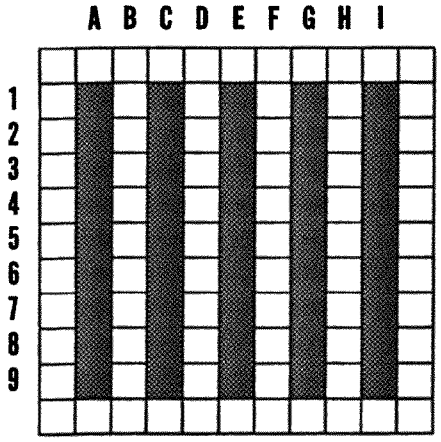


FIG. 19B

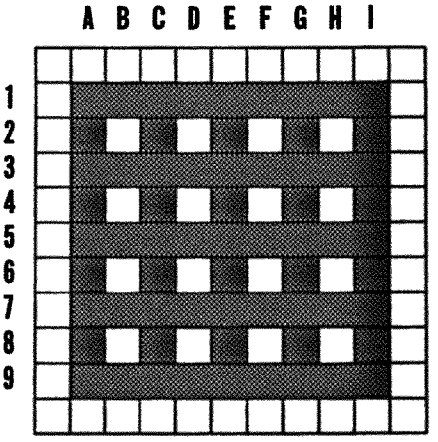


FIG. 19C

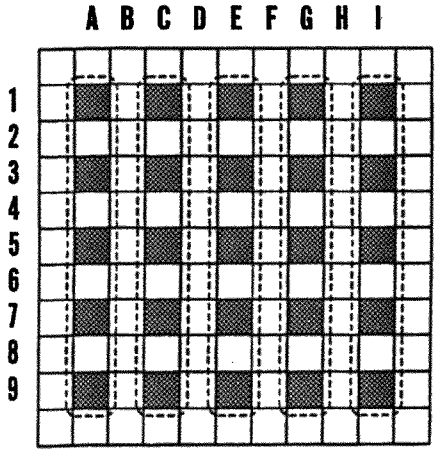


FIG. 19D

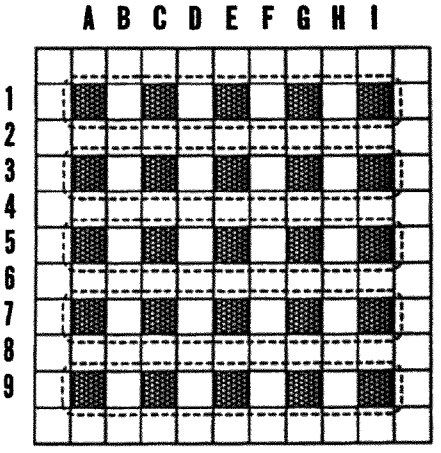


FIG. 19E

FIG. 20A

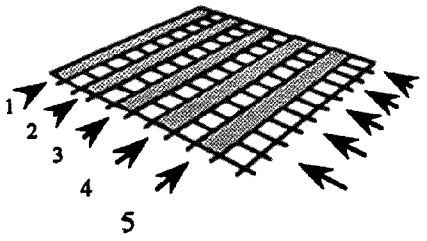
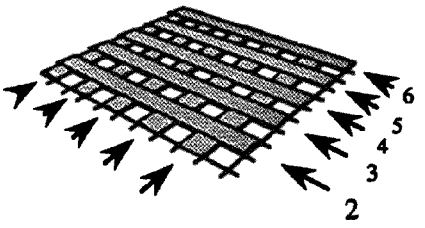


FIG. 20B



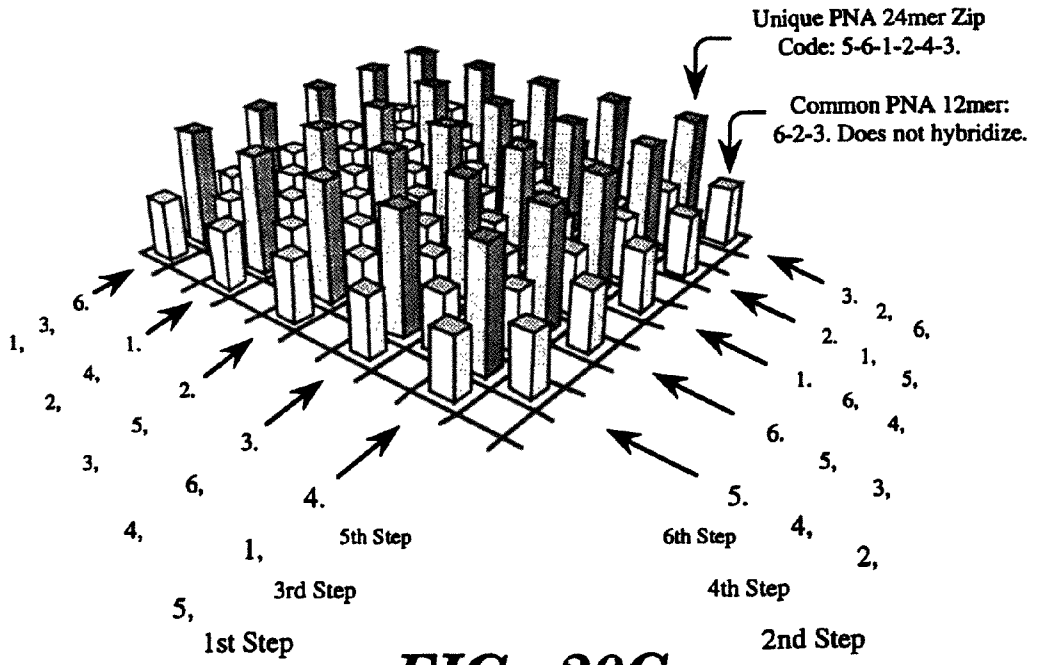


FIG. 20C

FIG. 21A



FIG. 21B



FIG. 21C



FIG. 21D

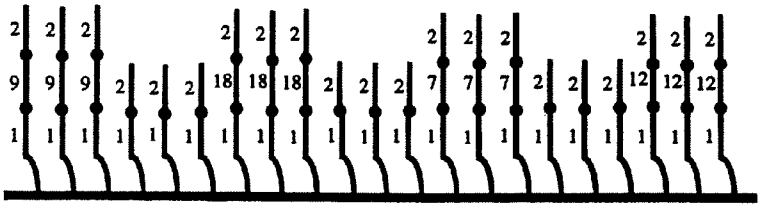


FIG. 21E

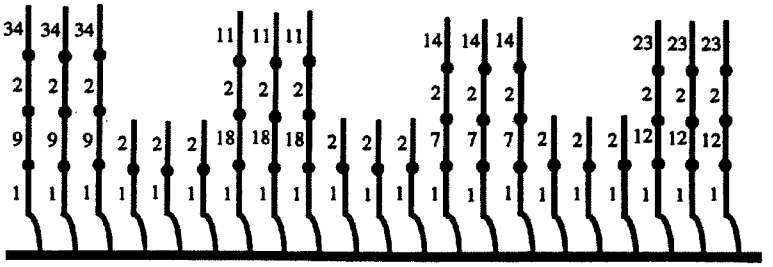
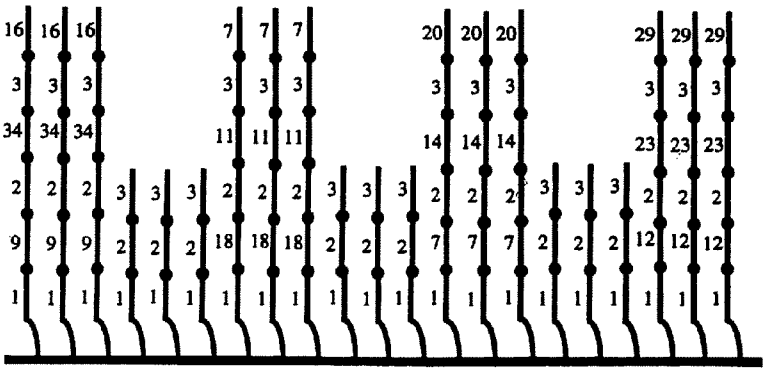


FIG. 21F



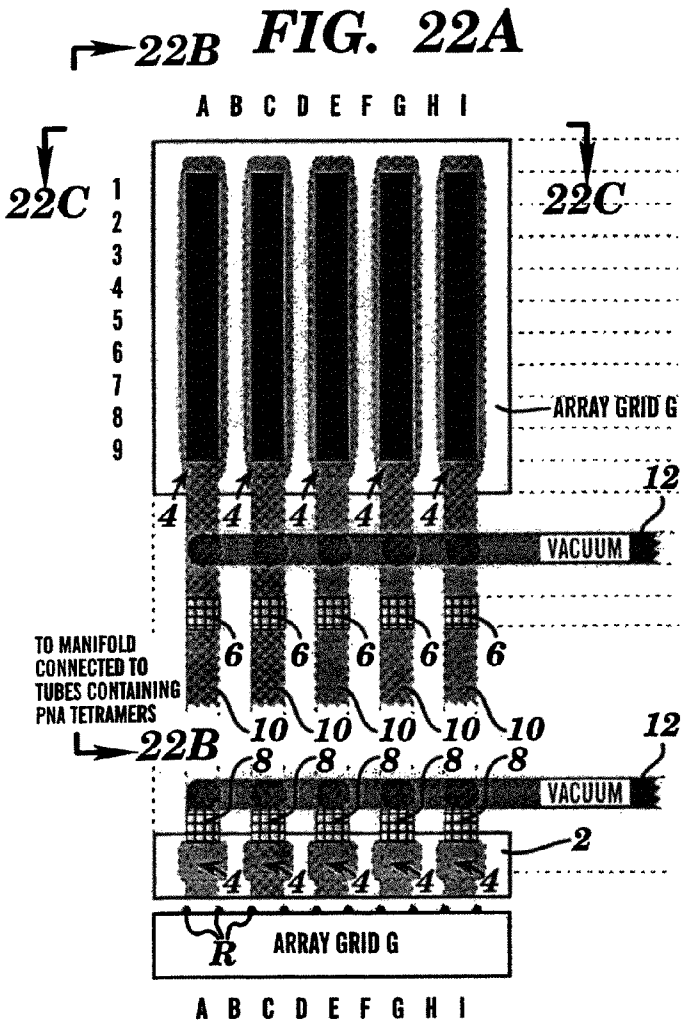


FIG. 22A

FIG. 22C

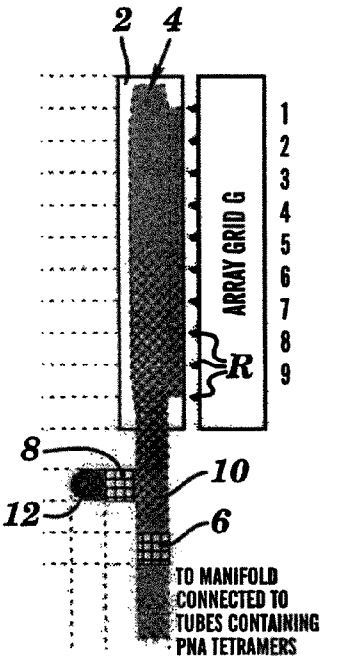


FIG. 22B

FIG. 23A

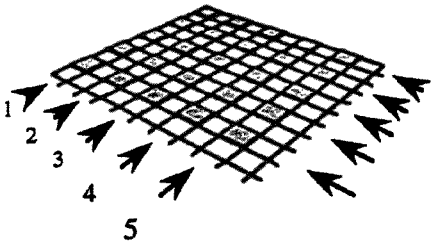
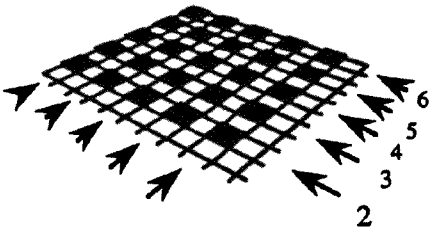


FIG. 23B



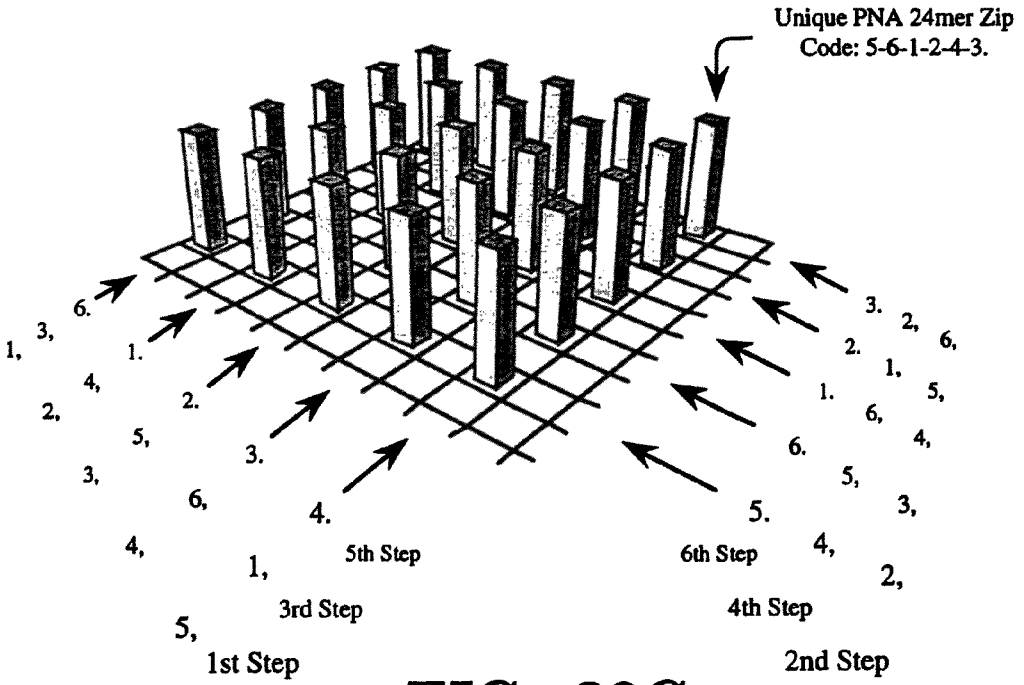


FIG. 23C

FIG. 24A

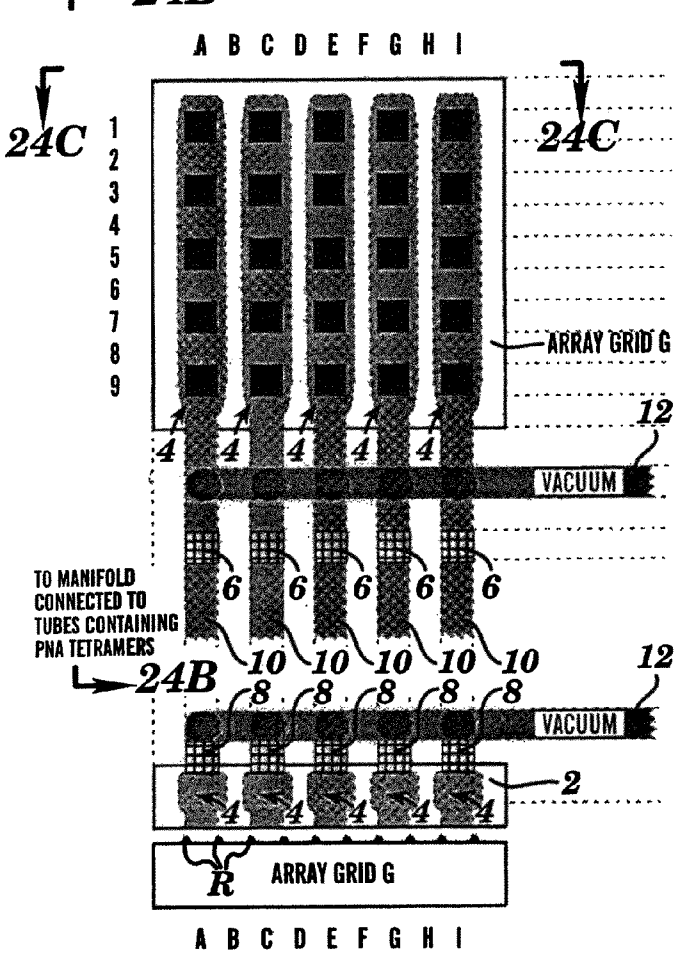


FIG. 24C

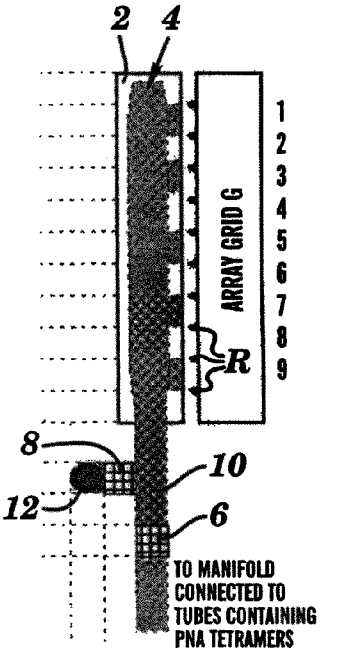


FIG. 24B

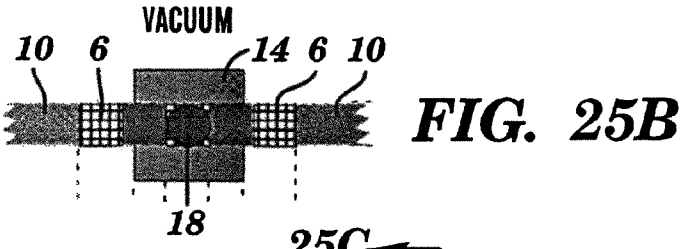


FIG. 25B

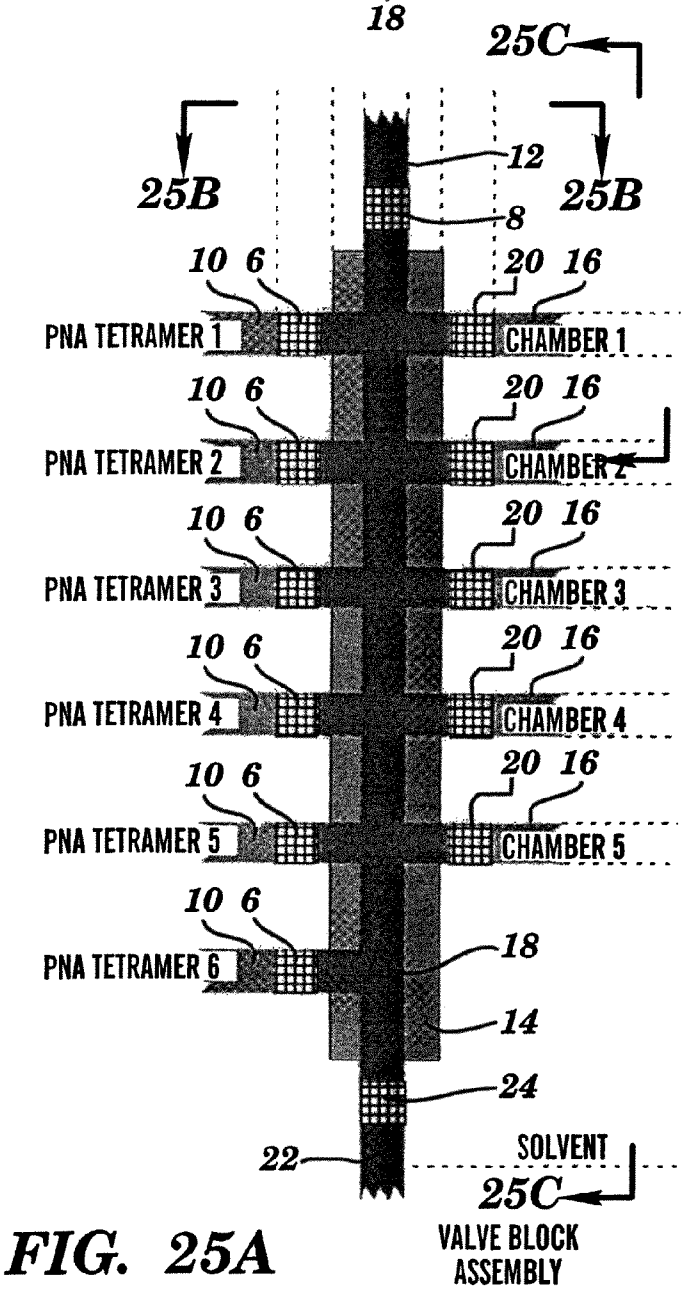


FIG. 25A

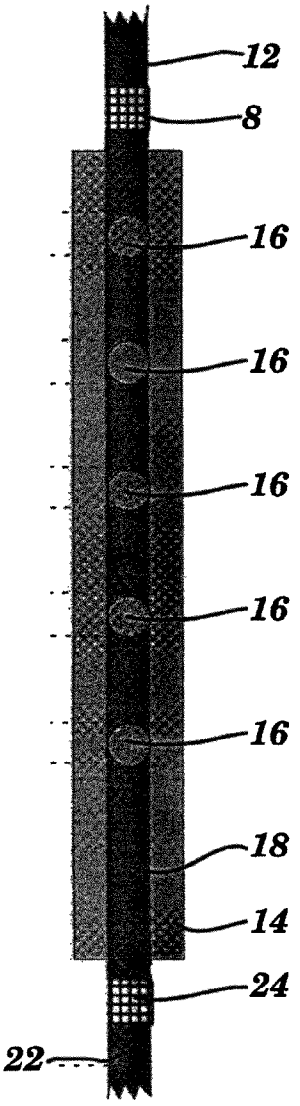


FIG. 25C

6 INPUTS AND 5 OUTPUTS

FIG. 26A

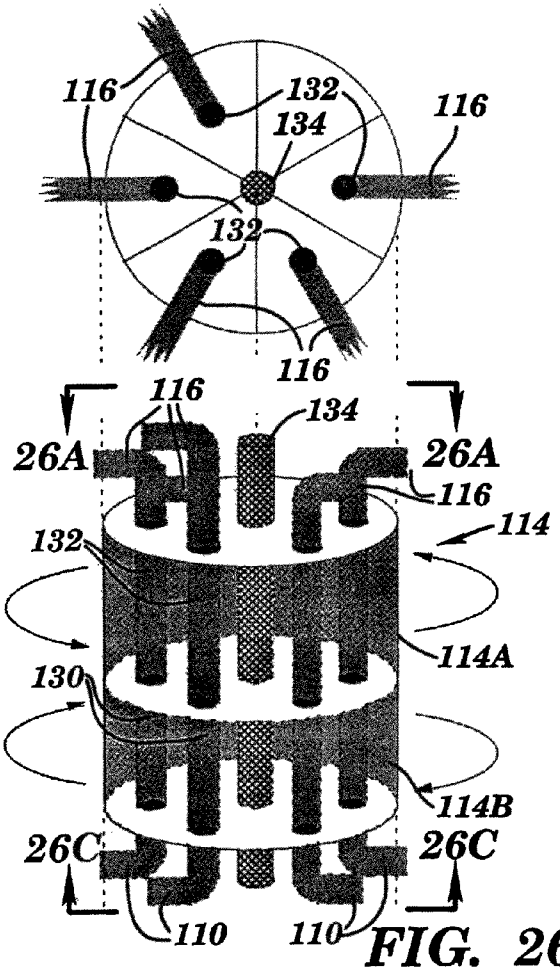


FIG. 26B

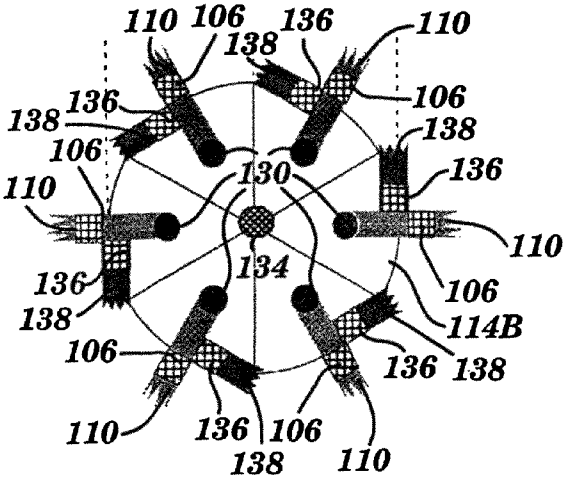


FIG. 26C

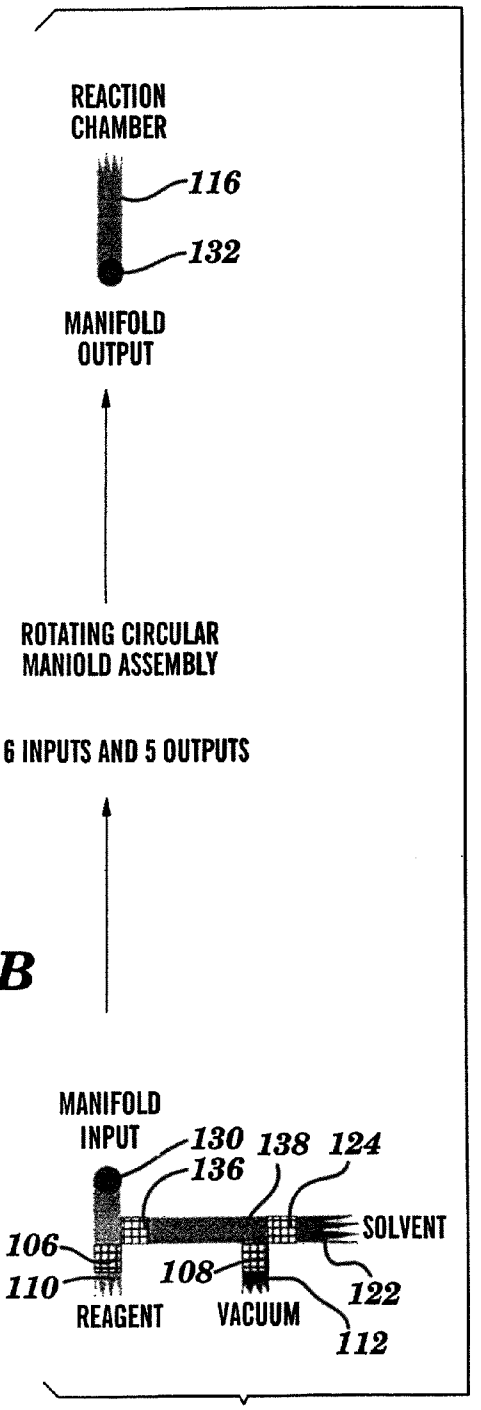


FIG. 26D

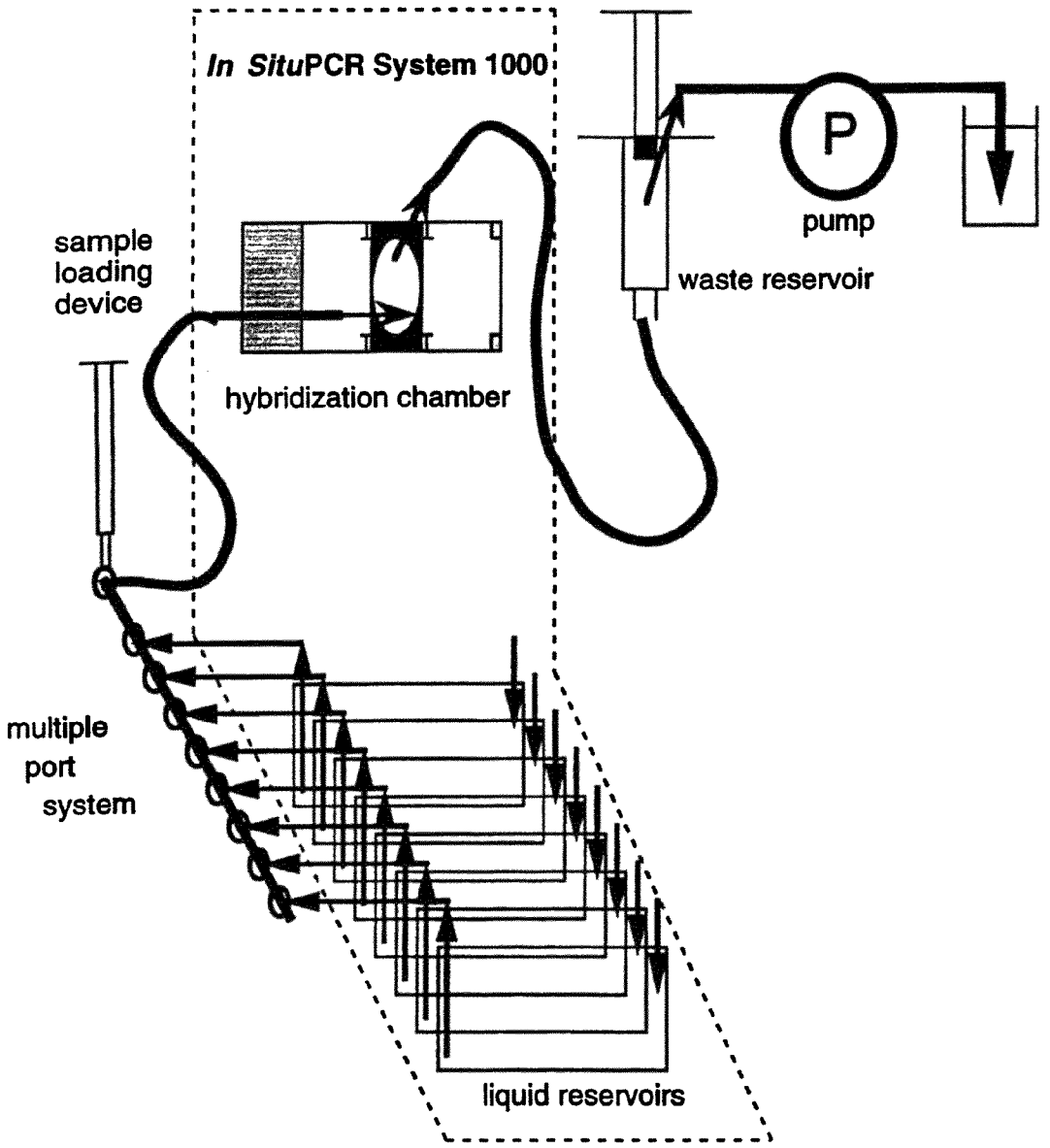

In SituPCR System 1000
P
pump
waste reservoir
sample loading device
hybridization chamber
multiple port system
liquid reservoirs

FIG. 27

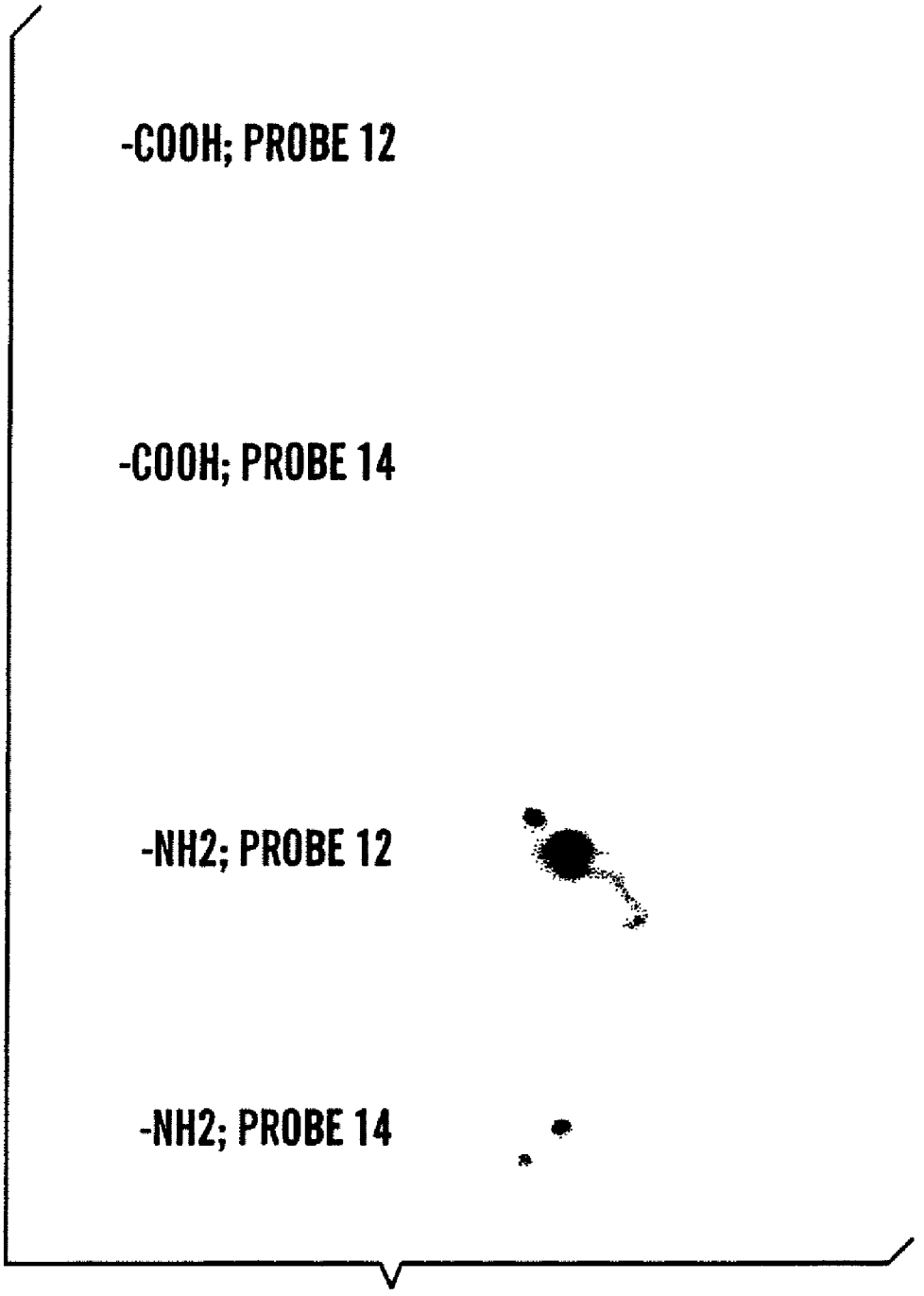


FIG. 28

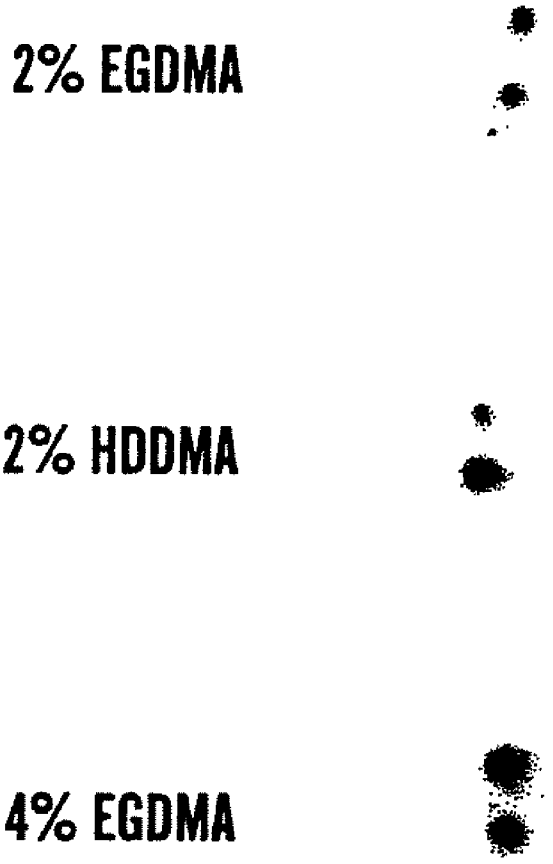


FIG. 29

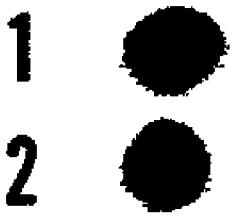


FIG. 30

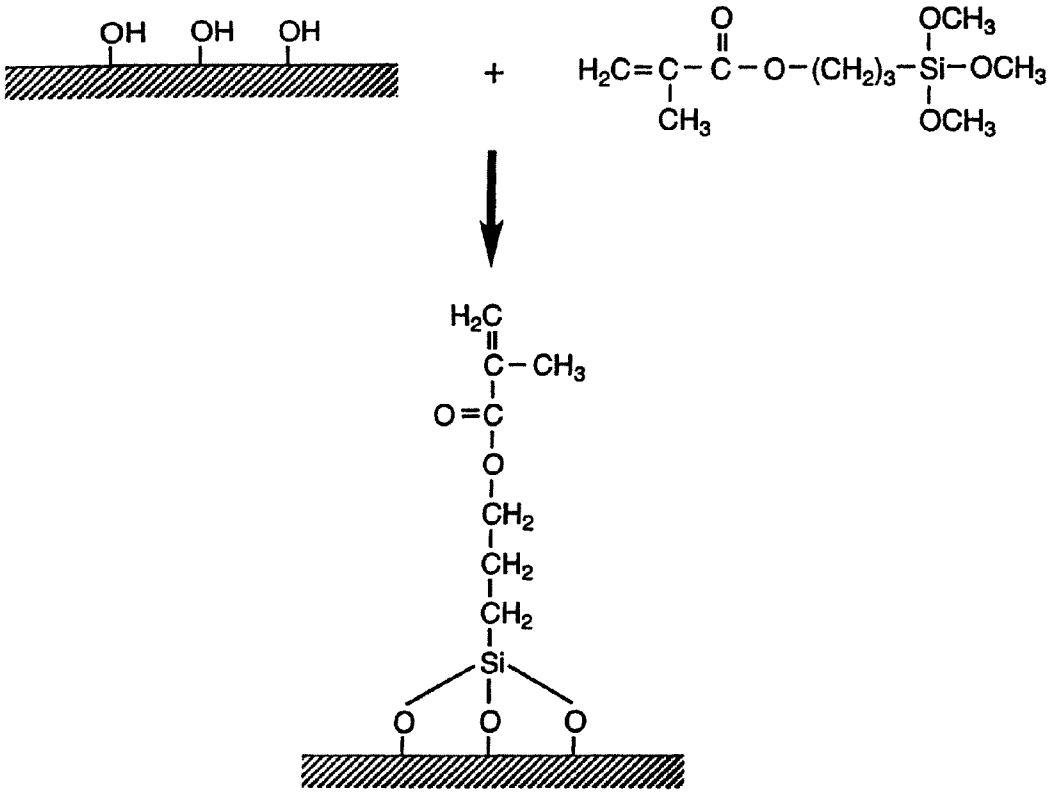

OH OH OH
+
O
OCH3
H2C=C-C-O-(CH2)3-Si-OCH3
CH3
OCH3
H2C
C-CH3
O=C
O
CH2
CH2
CH2
Si
O O O

FIG. 31

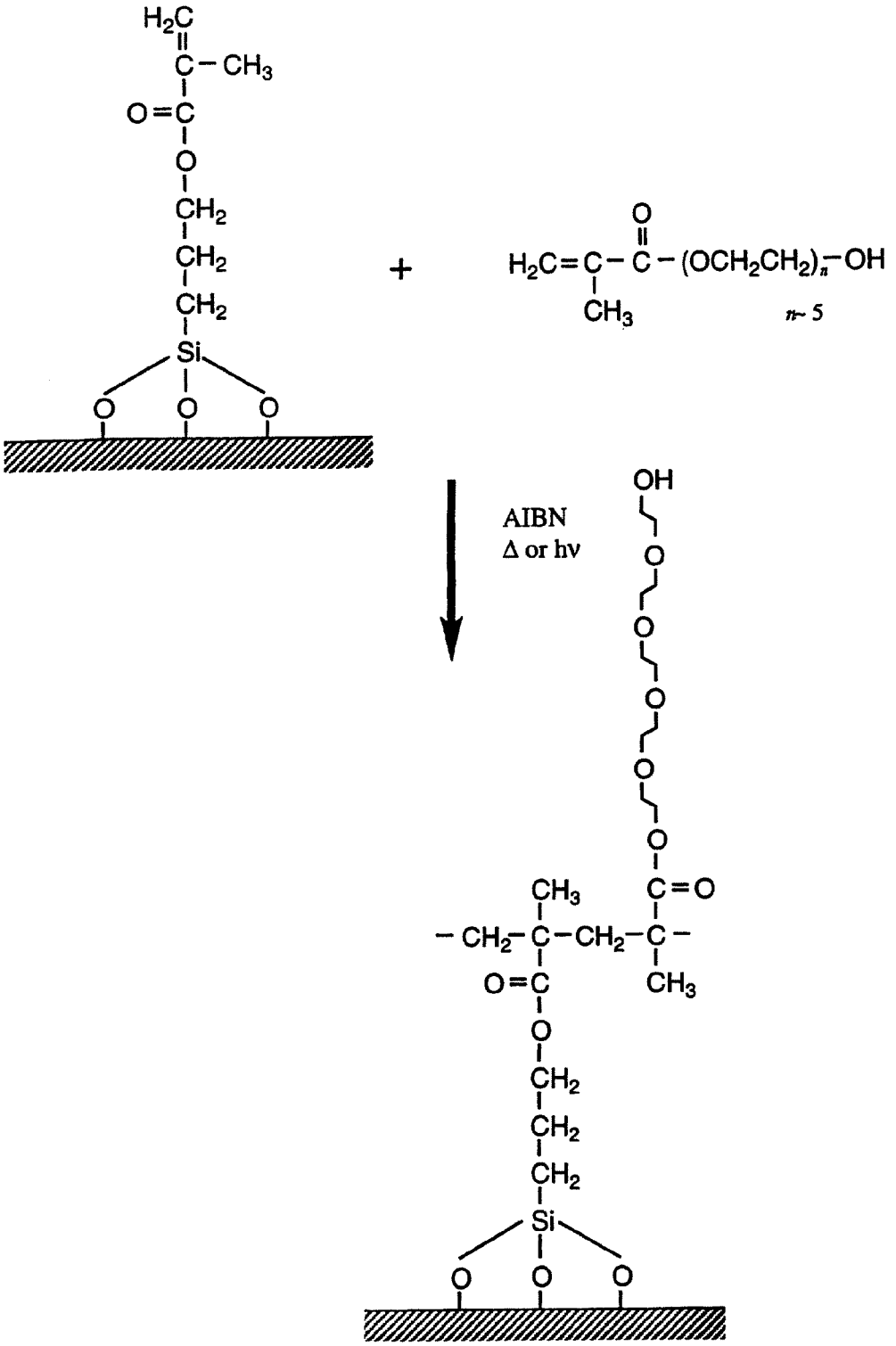


FIG. 32

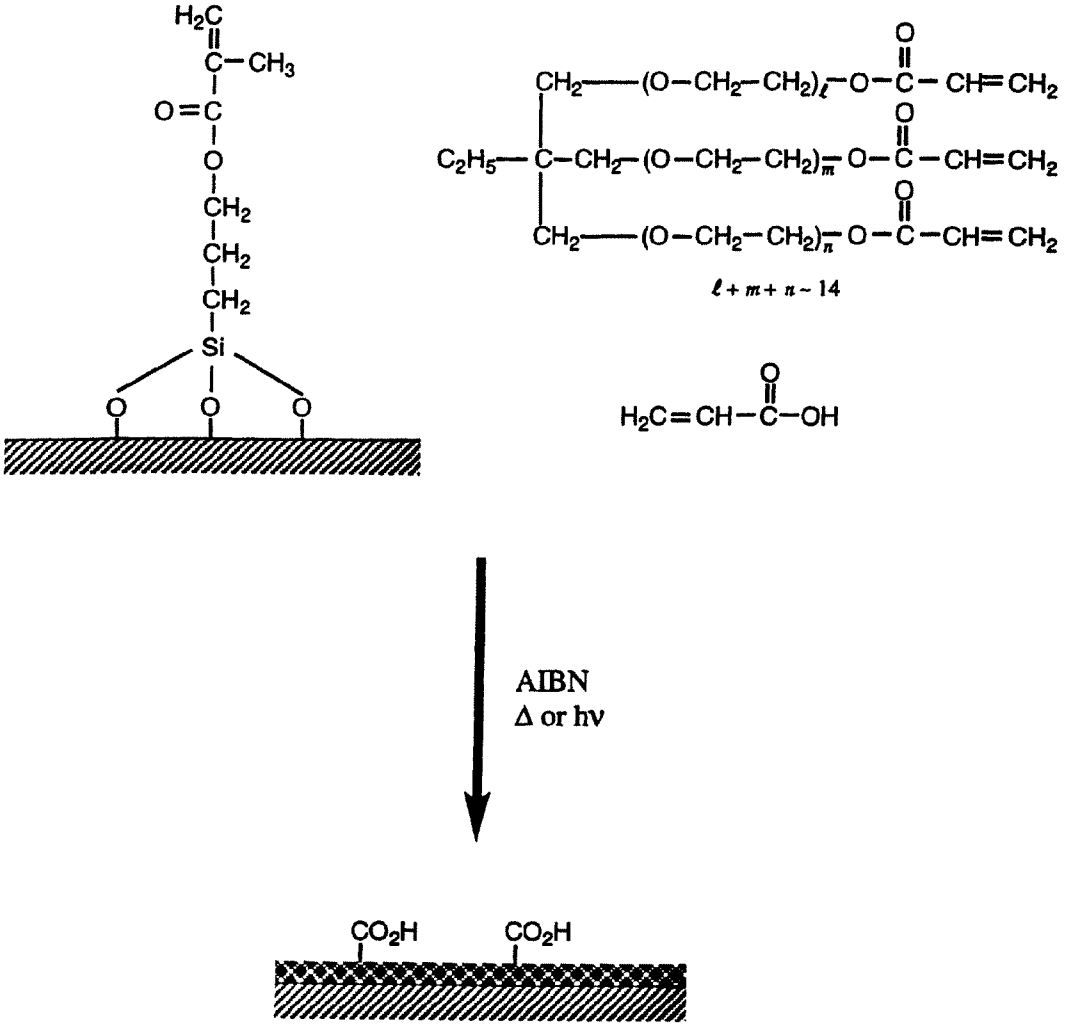


FIG. 33

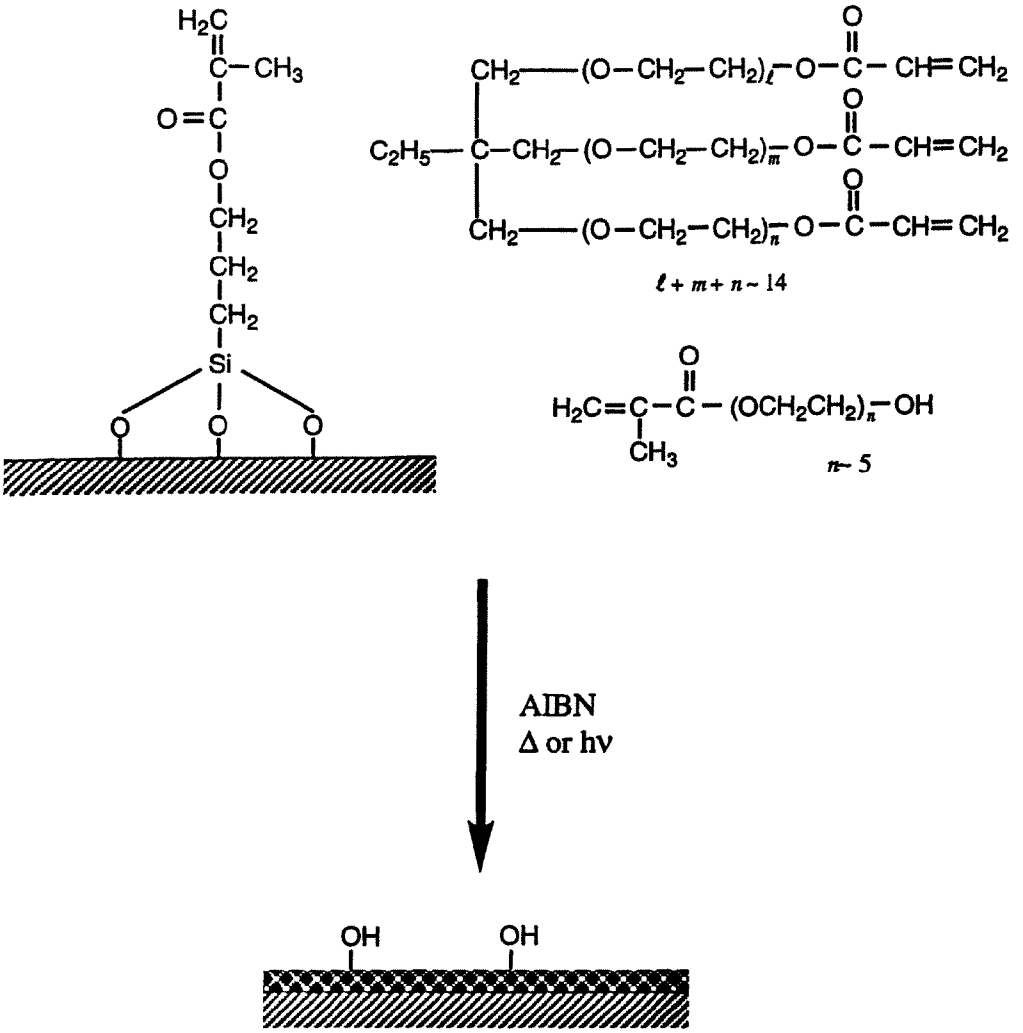


FIG. 34

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

The present application is a continuation of U.S. patent application Ser. No. 10/854,678, filed May 25, 2004, which is a continuation of U.S. patent application Ser. No. 08/794,851, filed Feb. 4, 1997, now U.S. Pat. No. 6,852,487, issued on Feb. 8, 2005, which claims the benefit of U.S. Provisional Patent Application Ser. No. 60/011,359, filed Feb. 9, 1996, which are hereby incorporated by reference in their entirety.

This invention was made with government support under grant number GM-41337-06 awarded by National Institutes of Health. The U.S. Government has certain rights in this invention.

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* 5:86-93 (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 polynucle-

otide. 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 FIGS. **13**A-C, 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**B, 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.

FIG. **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-G.

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-trimethoxy-silane.

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 transloca-

tions 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 4a, 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 4b, 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: 189-93 (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., "Thermophilic 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, yoghurt, 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 hybridiza-

tion would be in standard antiparallel 5' to 3' and 3' to 5'. Other modified sugar-phosphate backbones would be used in a similar fashion. FIG. **13**A 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). FIGS. **13**B-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.

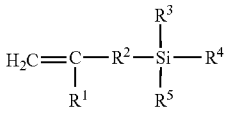
Surfaces on the solid substrate will usually, though not always, be composed of the same material as the substrate. Thus, the surface may be composed of any of a wide variety of materials, for example, polymers, plastics, ceramics, polysaccharides, silica or silica-based materials, carbon, metals, inorganic 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);

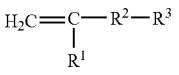
R^3 is an O-alkyl, alkyl, or halogen group;

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, COCl, 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.

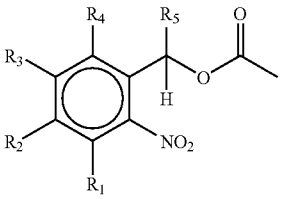
The linker molecules can be attached to the substrate via carbon-carbon bonds using, for example, (poly)tri-fluorochloroethylene surfaces or, preferably, by siloxane bonds (using, for example, glass or silicon oxide surfaces). Siloxane bonds with the surface of the substrate may be formed in one embodiment 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 oligo-tryptophanyl 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 refer-

ence); 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,* 13:1008-1017 (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).

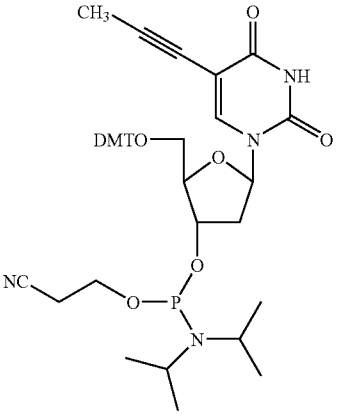
Although considerable progress has been made in constructing oligonucleotide arrays, some containing as many as 256 independent addresses, these procedures are less preferred, for detecting specific DNA sequences by hybridizations. More particularly, arrays containing longer oligonucleotides can currently 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.

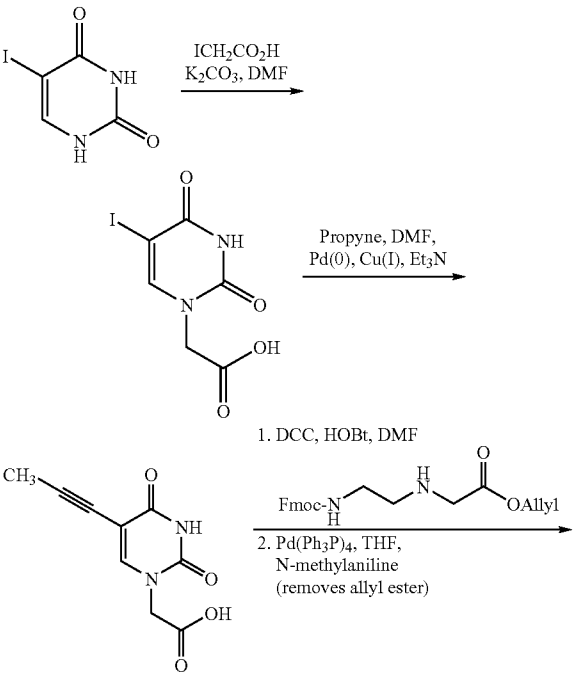
It is desirable to produce a solid support with a good loading of oligonucleotide or PNA oligomer in a relatively small, but well-defined area. Current, commercially available fluorescent imagers can detect a signal as low as 3 attomoles per 50 μm square pixel. Thus, a reasonable size address or "spot" 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.

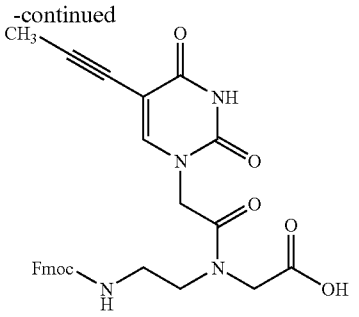
The melting temperature of DNA/DNA duplexes can be estimated as [4n(G·C)+2m(A·T)]° C. Oligonucleotide capture can be optimized by narrowing the T_m difference between duplexes formed by capture oligonucleotides and the complementary addressable array-specific portions hybridized to one another resulting 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):



-continued



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 **A1**, E**1**, I**1**, M**1**, A**5**, E**5**, I**5**, M**5**, A**9**, E**9**, I**9**, M**9**, A**13**, E**13**, I**13**, 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

TABLE 1-continued

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
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	Sequences (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 FIG. **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
5'-ATCG GGTA GGTA ACCT TGCG TGCG-3' (SEQ ID NO: 7)

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

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
5'-ATCG GGTA GGTA ACCT TGCG   (SEQ ID NO: 9)
TGCG-3'

Zip 14 (4-4-6-6-3-1) = 24 mer
5'-GGTA GGTA ACCT ACCT CAGC   (SEQ ID NO: 10)
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
5'-ATCG GGTA GGTA ACCT TGCG   (SEQ ID NO: 11)
TGCG-3'

Zip 3 (3-6-5-2-2-3) = 24 mer
5'-CAGC ACCT GACC ATCG ATCG   (SEQ ID NO: 12)
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 24-mer 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-G). 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 third round synthesis of PNA rows. The first row contains tetramer 3. FIG. **21**F shows the structure of the array after third round synthesis of columns, adding tetramers 16, 7, 20, 29. Note that all 24-mer 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 at 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 (FIG. **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-**24**B and line **24**C-**24**C 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 (FIG. **25**A-C) will route each tetramer to the appropriate row. Alternatively, a cylindrical manifold (FIG. **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**. FIG. **25**A is a top view of valve block assembly **14**, while side views FIGS. **25**B and **25**C are, respectively, taken along line **25**B-**25**B and line **25**C-**25**C of FIG. **25**A. 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 Enzymol.*, 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 non-specifically 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 25G ¾ 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 21G 3/4" 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 21 G 3/4" 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 oligonucleotide 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.

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)

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 4A

Quantification of captured oligonucleotide probe 12

Functional group on slide	Probe	Oligonucleotide probe at capture oligonucleotide 12 (pic)*	(amol)	Oligonucleotide probe at capture oligonucleotide 14 (pic)*	(amol)	Average cross reactivity
—COOH	12	105,333	9.0			0.37
—COOH	12	55,957	4.8			

TABLE 4A-continued

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			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 mismatches, 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 nmol/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 on glass slide	Probe	Oligonucleotide probe at capture oligonucleotide 14 (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 1.5 ml 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

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	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

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	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

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	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. Jönsson, 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 glove-box 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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cgcacgcaag gttacctacc cgat 24

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<223> OTHER INFORMATION: oligonucleotide

<400> SEQUENCE: 16

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What is claimed:

1. A method for identifying a plurality of target nucleotide sequences in a sample, said method comprising:

providing a sample potentially containing a plurality of target nucleotide sequences;

providing a plurality of composite oligonucleotide probes characterized by having a target-specific portion coupled to a zip code portion, wherein the zip code portions of the composite oligonucleotide probes comprise a nucleotide sequence having greater than sixteen nucleotides that differs for each target-specific portion and hybridizes to their complements under uniform hybridization conditions, wherein the nucleotide sequence of a complement to one zip code portion differs from the nucleotide sequence of a complement to another zip code portion, when aligned to each other, by at least 25% of the nucleotides, to minimize cross-hybridization of one zip code portion to a complement of another zip code portion;

attaching one or more labels to one or more of the plurality of composite oligonucleotide probes if their respective complementary target nucleotide sequences are present in the sample;

providing a plurality of secondary oligonucleotide probes, each comprising a nucleotide sequence complementary to that of a zip code portion of the composite oligonucleotide probes, wherein each zip code portion hybridizes to its complementary secondary oligonucleotide probe in a base-specific manner; and

detecting and distinguishing labeled products resulting from the hybridization of the target specific portions of the composite oligonucleotide probes to complementary target nucleotide sequences, if present, in the sample, and hybridization of the zip code portions of the composite oligonucleotide probes to their respective complementary secondary oligonucleotide probes, thereby identifying the presence of one or more target nucleotide sequences in the sample.

2. A method according to claim 1, wherein the plurality of secondary oligonucleotide probes are coupled to a solid support.

3. A method according to claim 2, wherein different secondary oligonucleotide probes are coupled to the solid support at different positions to detect different target nucleotide sequences.

4. A method according to claim 1, wherein the plurality of secondary oligonucleotide probes are coupled to beads.

5. A method according to claim 1, wherein the plurality of composite oligonucleotide probes are coupled to a solid support.

6. A method according to claim 5, wherein different composite oligonucleotide probes are coupled to the solid support at different positions to detect different target nucleotide sequences.

7. A method according to claim 1, wherein the plurality of composite oligonucleotide probes are coupled to beads.

8. A method according to claim 1 further comprising:

amplifying the target nucleotide sequences in the sample.

9. A method according to claim 8, wherein said amplifying is carried out by subjecting the sample to a polymerase chain reaction process.

10. A method according to claim 9, wherein said polymerase chain reaction process is carried out with DNA polymerase.

11. A method according to claim 1 further comprising:

blending the sample, the one or more labels, and the plurality of composite oligonucleotide probes to form a blend and

subjecting the blend to one or more hybridization treatments suitable for the target-specific portions of the composite oligonucleotide probes to hybridize in a base-specific manner to their respective target nucleotide sequences, if present in the sample, and ligate to their respective labels to form primary products, each product comprised of the composite oligonucleotide probe, the target nucleotide sequence hybridized in a base-specific manner to its respective composite oligonucleotide probe, and the one or more labels attached to the composite oligonucleotide probe only if its complementary target nucleotide sequence is present in the sample.

12. A method according to claim 11 further comprising:

providing a solid support with the plurality of secondary oligonucleotide probes attached at particular sites and

contacting the primary products with the solid support under uniform hybridization conditions suitable for hybridization in a base-specific manner of the zip code portions to their complementary secondary oligonucleotide probes under conditions that minimize cross hybridization between a zip code portion and a non-complementary secondary oligonucleotide probe, said detecting and distinguishing comprising:

detecting the labeled primary products captured on the solid support at particular sites, thereby indicating the presence of target nucleotide sequences in the sample.

13. The method according to claim 12, wherein each secondary oligonucleotide comprises 20-25 nucleotides.

14. The method according to claim 12, wherein the zip code portions hybridize to their complementary secondary oligonucleotides under highly stringent uniform hybridization conditions.

15. A method according to claim 11, wherein the blend comprises an enzyme capable of attaching the one or more labels to the composite oligonucleotide probes.

16. A method according to claim 15, wherein the enzyme is a ligase.

17. A method according to claim 11, wherein the one or more labels comprise oligonucleotide probes having a target-specific portion coupled to a reporter group and said subjecting comprises carrying out one or more ligase detection reaction cycles to form a ligation product comprising (a) the zip code portion, (b) the target-specific portions, and the reporter group if their respective target nucleotide sequence is present in the sample, said method further comprising:

providing a solid support with the plurality of secondary oligonucleotides immobilized at particular sites and

contacting the blend after carrying out said ligase detection reaction with the solid support under uniform hybridization conditions effective to hybridize the zip code portions of the ligation product to the complementary secondary oligonucleotides in a base-specific manner, thereby capturing the ligation products on the solid support at the site with the complementary secondary oligonucleotide, said detecting and distinguishing comprising:

detecting the ligation products captured on the solid support at particular sites, thereby indicating the presence of target nucleotide sequences in the sample.

18. The method according to claim 17, wherein each secondary oligonucleotide comprises 20-25 nucleotides.

19. The method according to claim 17, wherein the zip code portions of the ligation products hybridize to their

complementary secondary oligonucleotides under highly stringent uniform hybridization conditions.

20. The method according to claim **17**, wherein each ligase detection reaction cycle comprises a denaturation treatment and a hybridization treatment, each being from about 30 seconds to about five minutes long.

21. The method according to claim **20**, wherein the denaturation treatment is at a temperature of about 80°-105° C.

22. The method according to claim **17**, wherein the ligase detection reaction is carried out for 2 to 50 cycles.

23. The method according to claim **17**, wherein total time for the ligase detection reaction is 1 to 250 minutes.

24. The method according to claim **17**, wherein the target-specific portions have a hybridization temperature of 20-85° C.

25. The method according to claim **17**, wherein the target-specific portions are 20 to 28 nucleotides long.

26. The method according to claim **17**, wherein the ligase detection reaction is carried out with a ligase selected from the group consisting of *Thermus aquaticus* ligase, *Thermus thermophilus* ligase, *E. coli* ligase, T4 ligase, and *Pyrococcus* ligase.

* * * * *

Exhibit K

US007893233B2

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

(10) **Patent No.: US 7,893,233 B2**
(45) **Date of Patent: Feb. 22, 2011**

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

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(21) Appl. No.: **12/725,230**

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(51) **Int. Cl.**
C07H 21/04 (2006.01)
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(52) **U.S. Cl.** **536/23.1**; 435/6; 536/24.31; 536/24.32

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

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(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.

27 Claims, 34 Drawing Sheets

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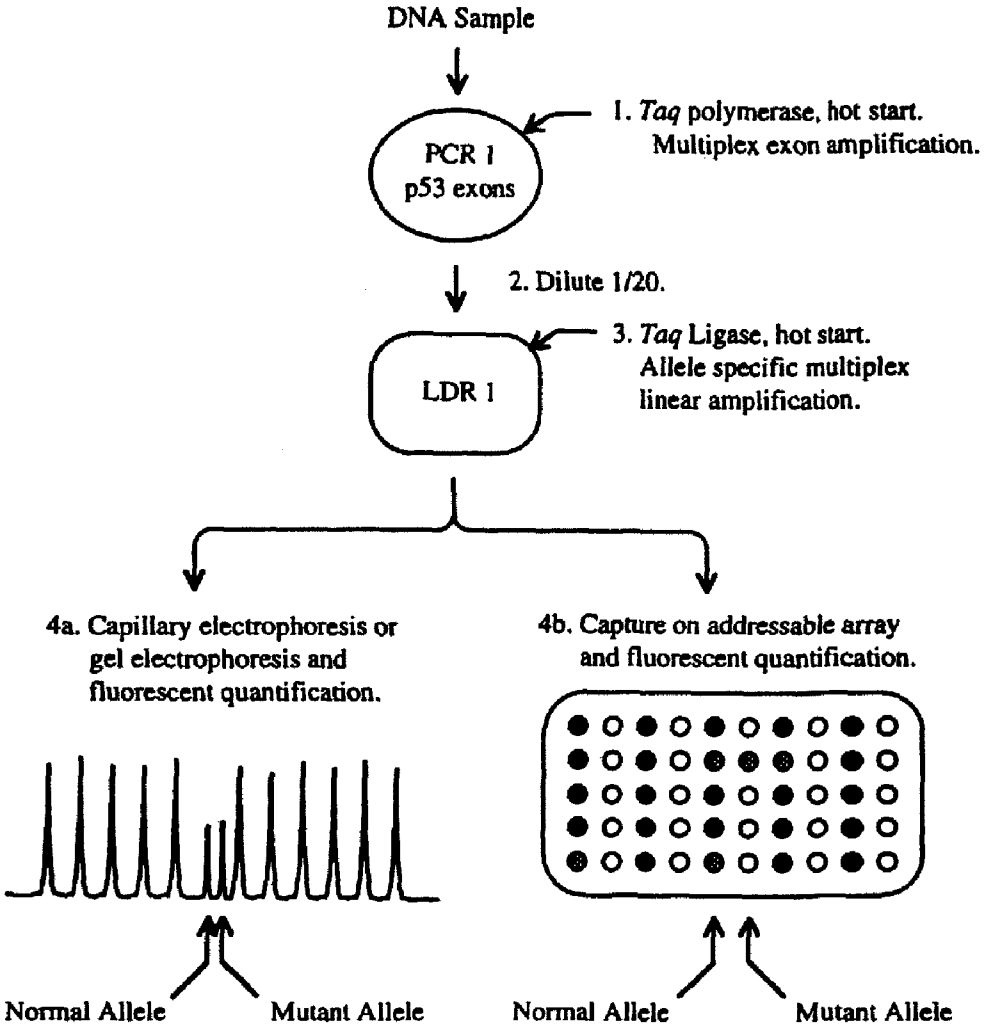

DNA Sample
1. Taq polymerase, hot start. Multiplex exon amplification.
PCR 1 p53 exons
2. Dilute 1/20.
3. Taq Ligase, hot start. Allele specific multiplex linear amplification.
LDR 1
4a. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
4b. Capture on addressable array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

FIG. 1

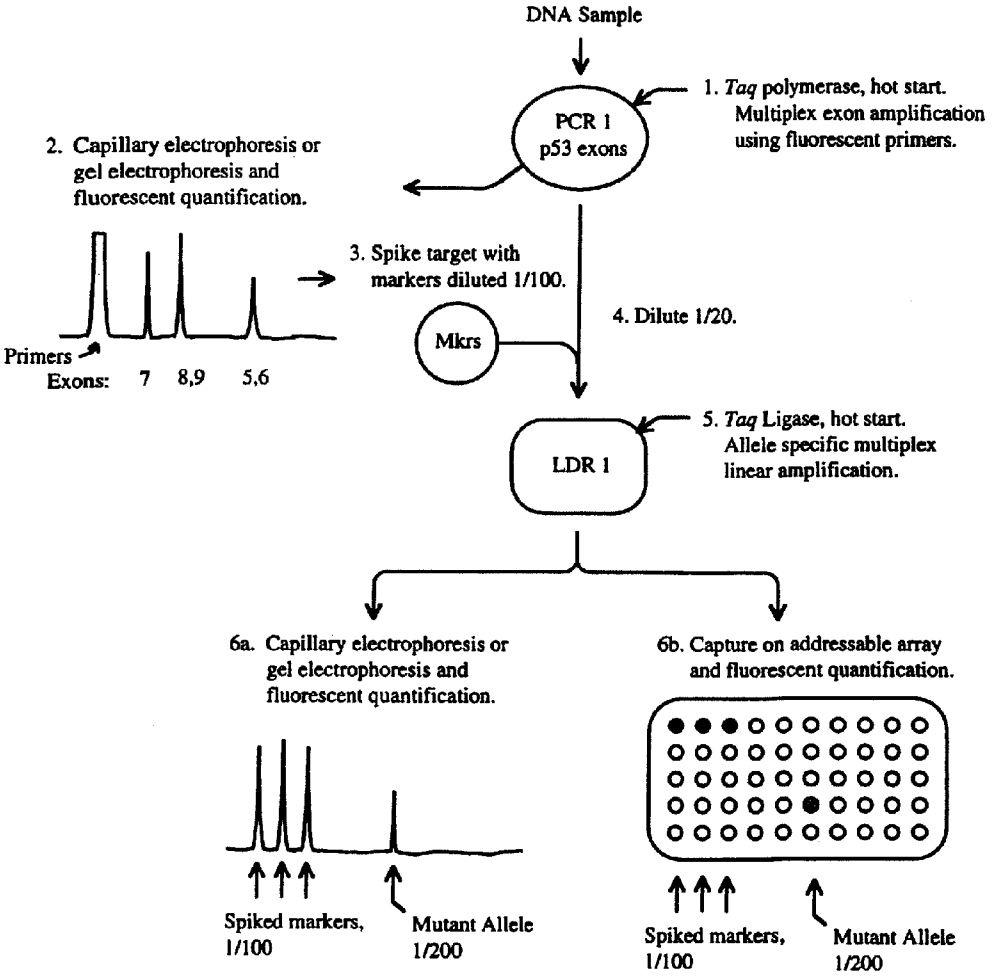

DNA Sample
1. Taq polymerase, hot start. Multiplex exon amplification using fluorescent primers.
PCR 1 p53 exons
2. Capillary electrophoresis or gel electrophoresis and fluorescent quantification.
3. Spike target with markers diluted 1/100.
Mkrs
4. Dilute 1/20.
Primers
Exons: 7 8,9 5,6
LDR 1
5. Taq Ligase,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

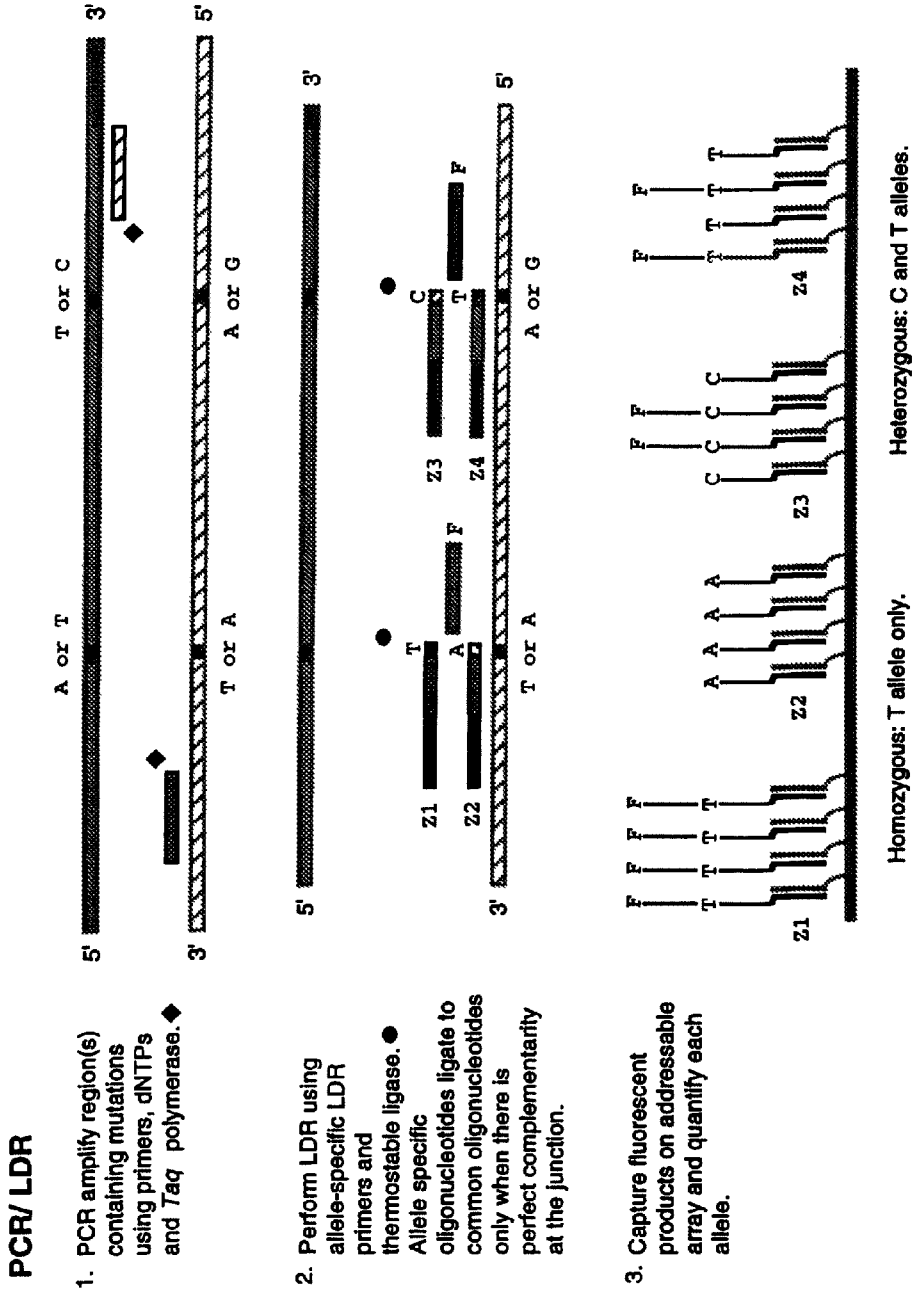

PCR/ LDR
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
3. 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

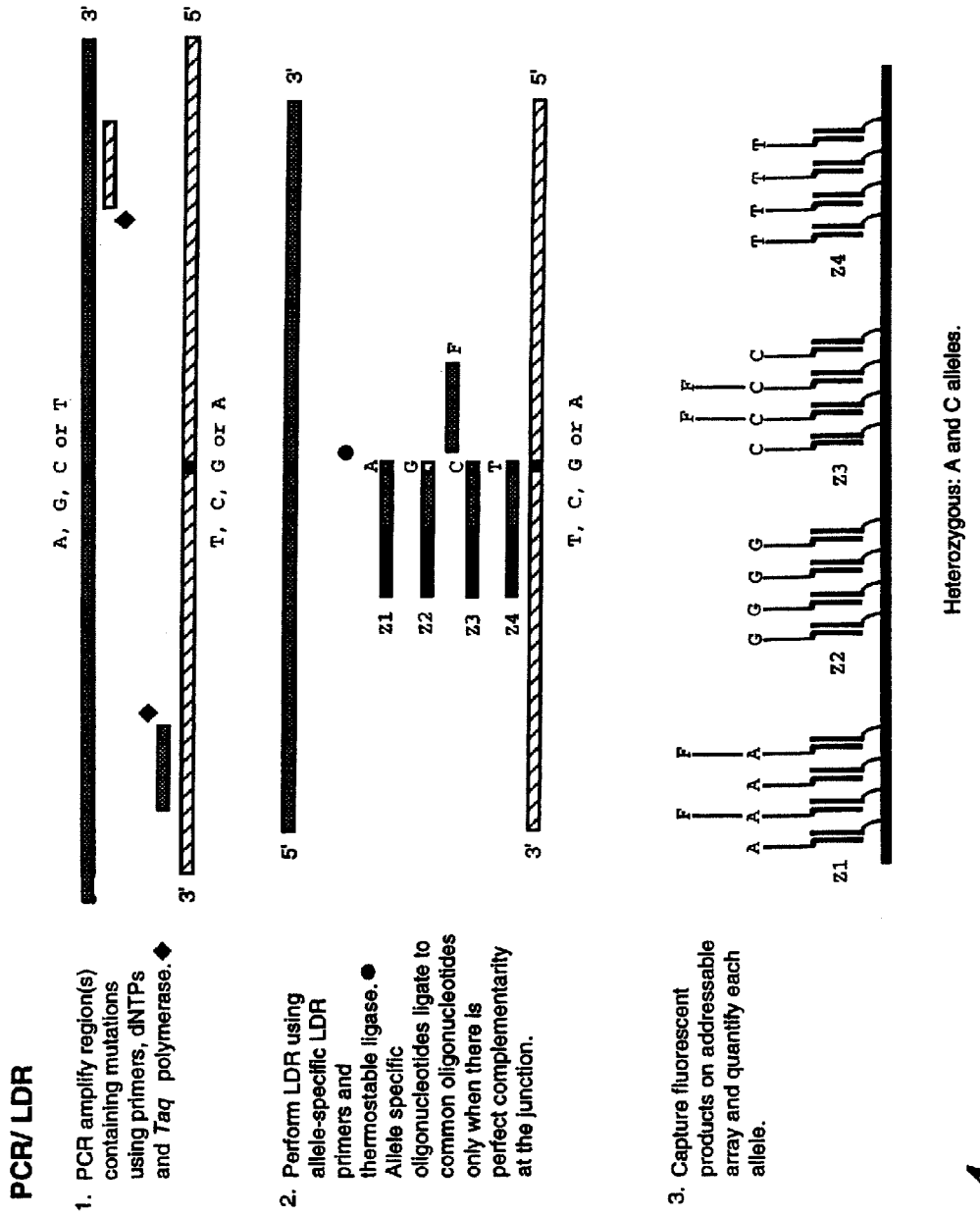

PCR/ LDR
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
3. Capture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

FIG. 4

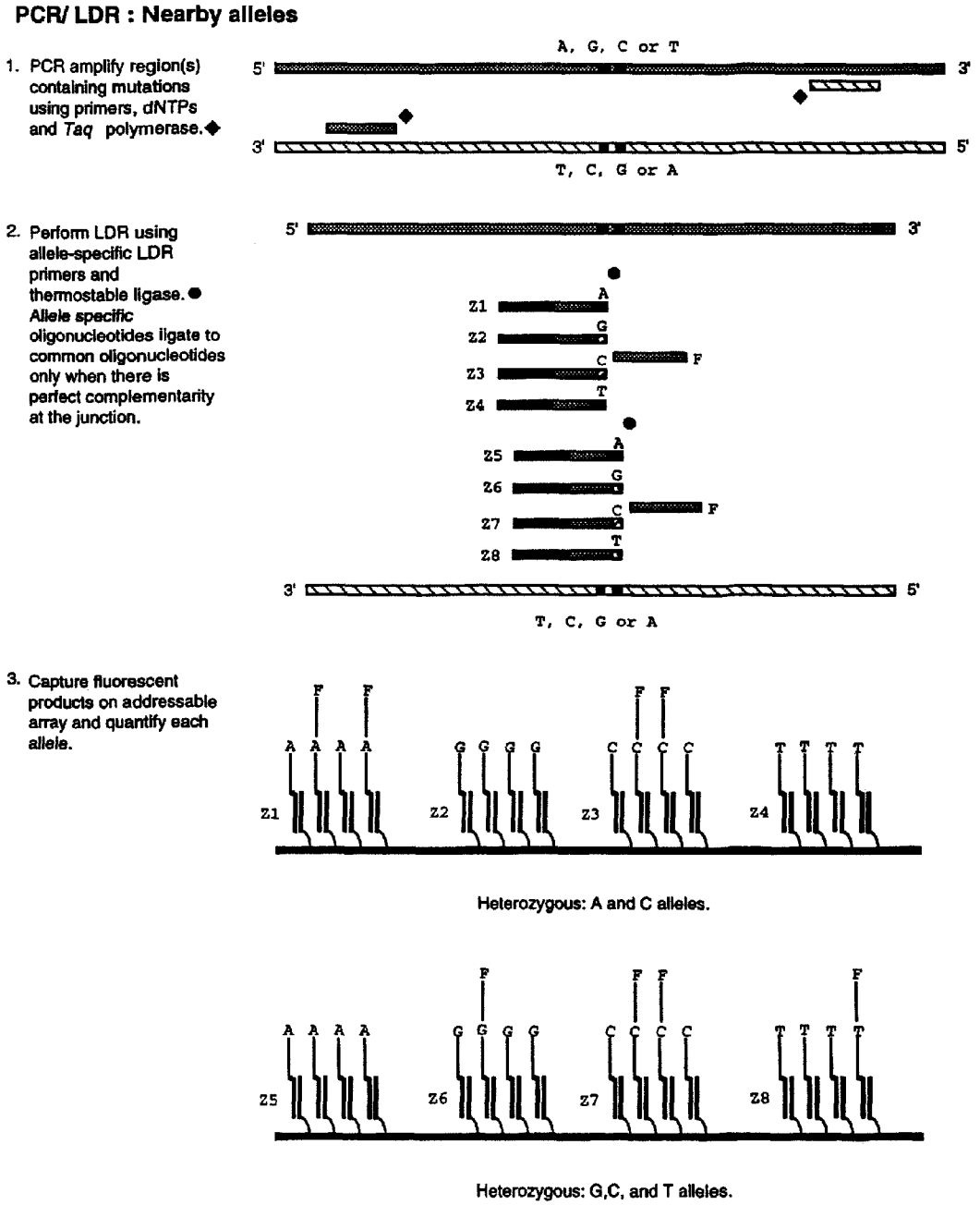

PCR/ LDR : Nearby alleles
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
A, G, C or T
5'
3'
3'
5'
T, C, G or A
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate to common oligonucleotides only when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

FIG. 5

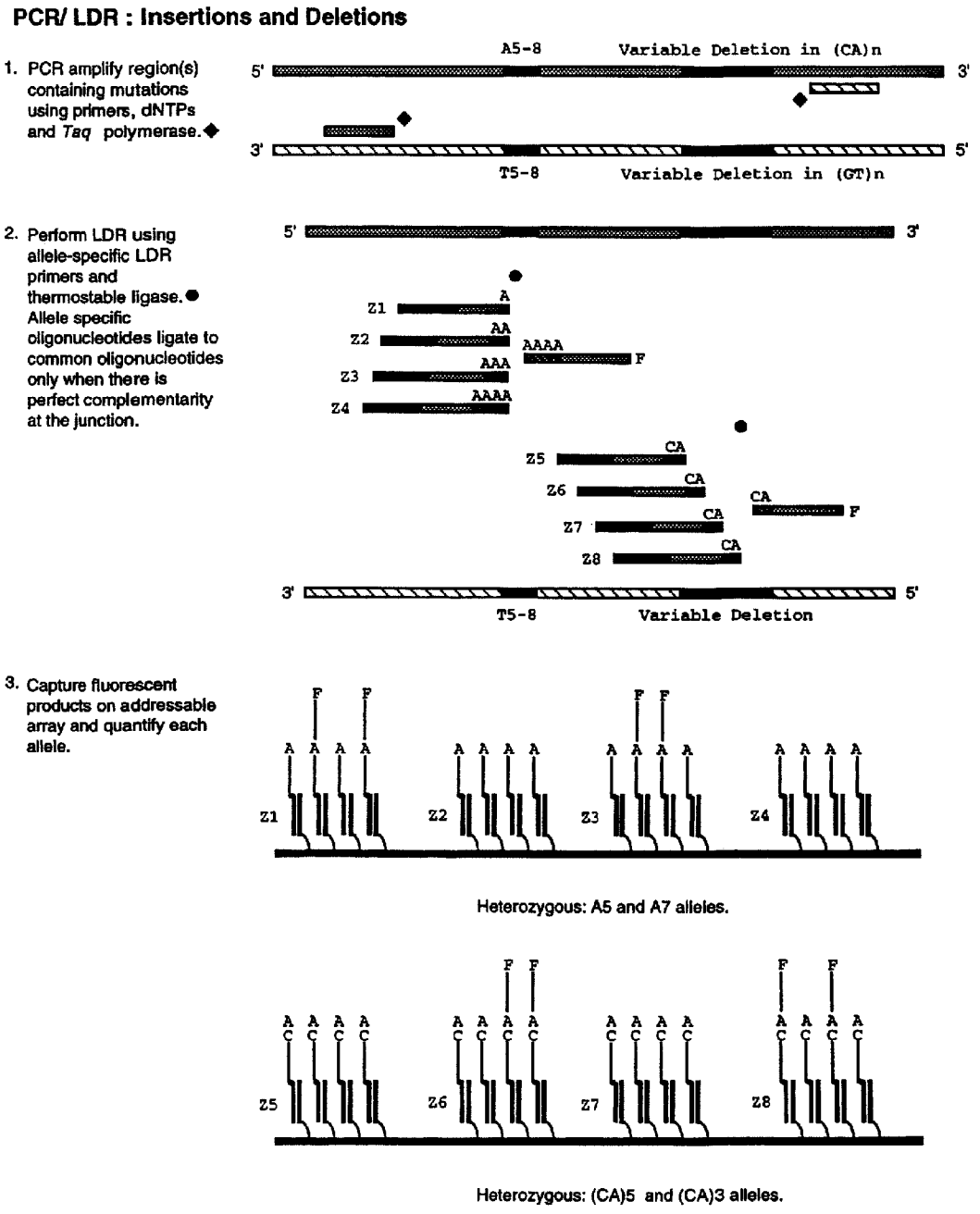

PCR/ LDR : Insertions and Deletions
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
A5-8
Variable Deletion in (CA)n
5'
3'
T5-8
Variable Deletion in (GT)n
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate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

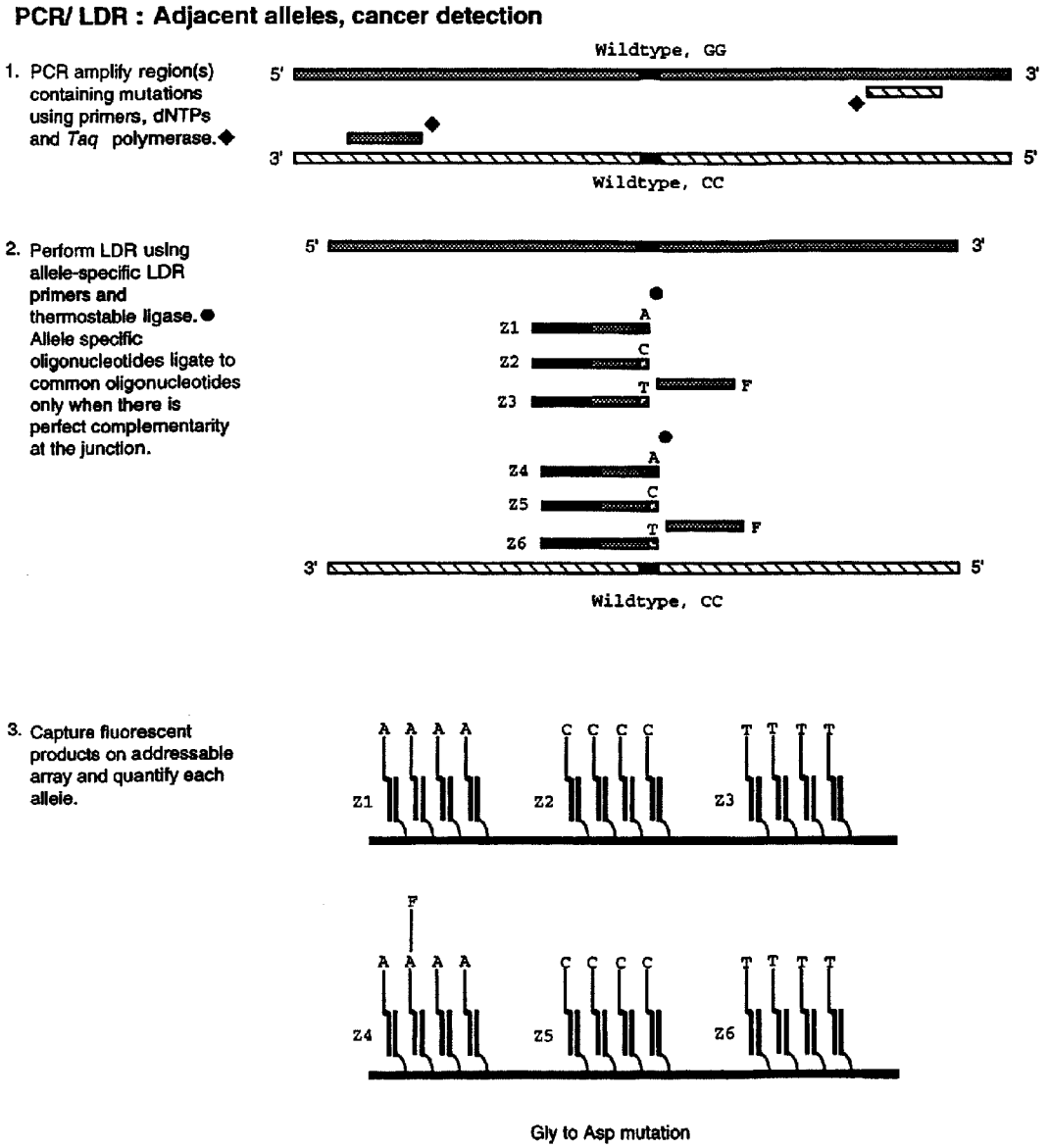

PCR/ LDR : Adjacent alleles, cancer detection
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
Wildtype, GG
5'
3'
3'
5'
Wildtype, CC
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate to common oligonucleotides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

FIG. 7

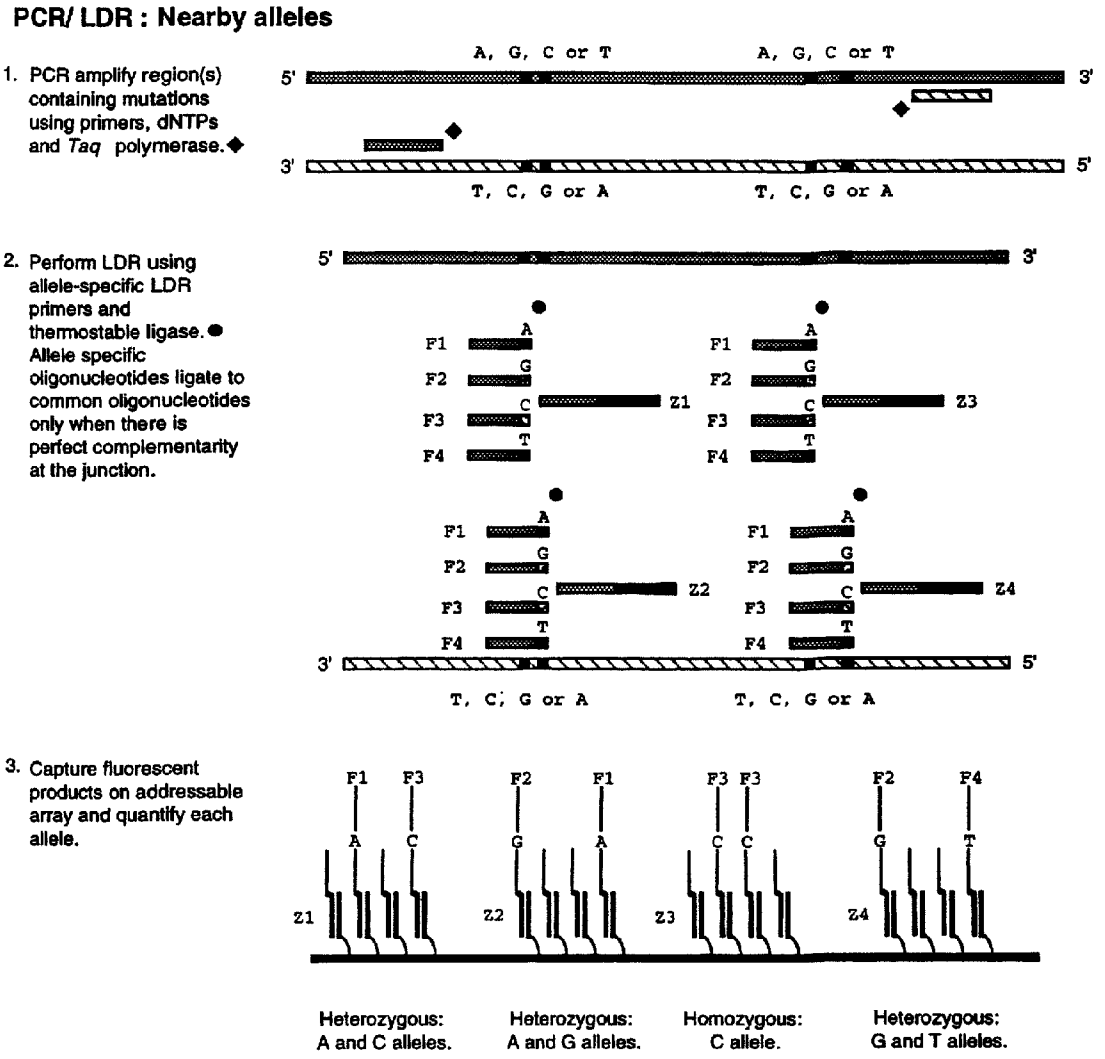

PCR/ LDR : Nearby alleles
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
A, G, C or T
A, G, C or T
5'
3'
3'
5'
T, C, G or A
T, C, G or A
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common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

FIG. 8

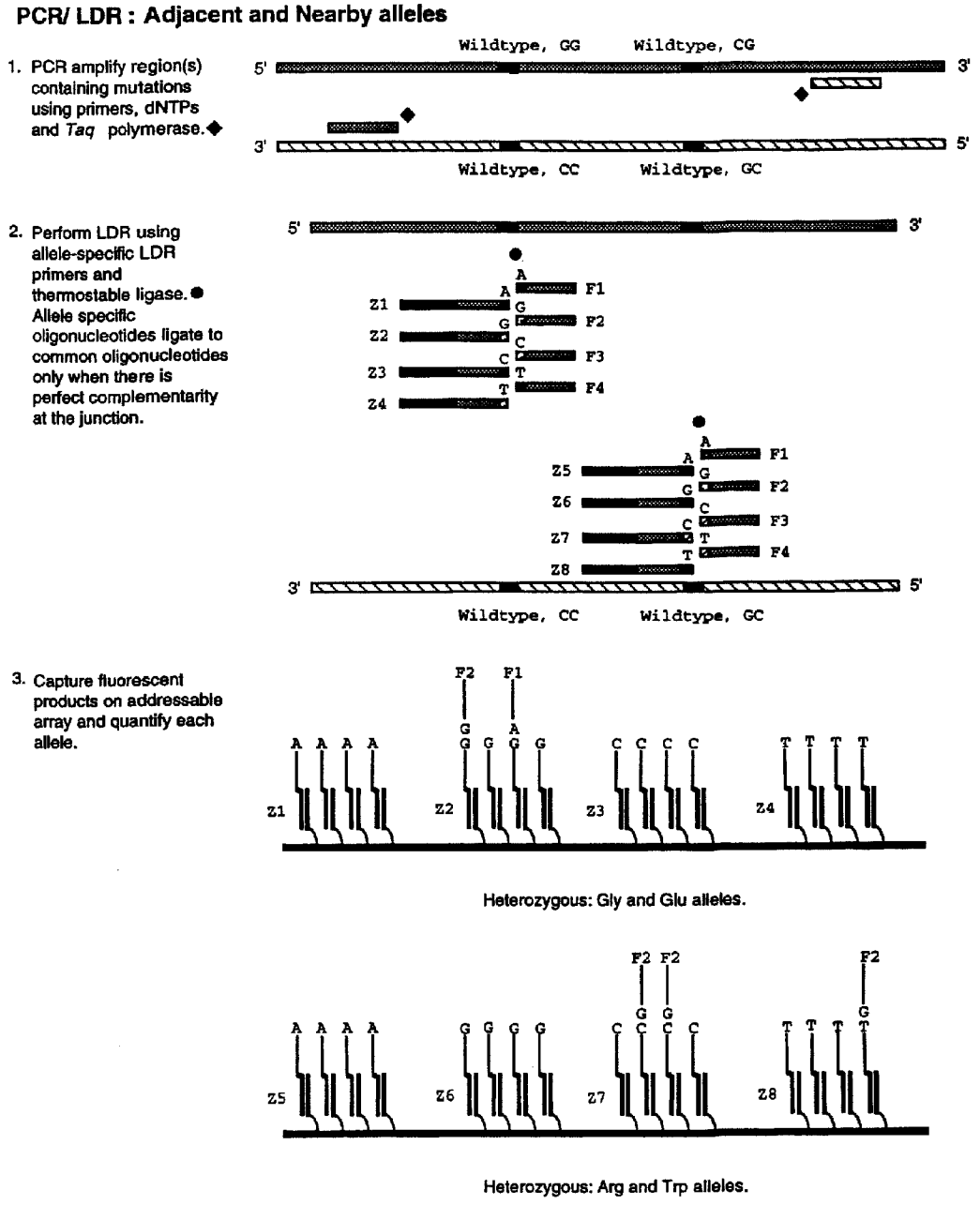

PCR/ LDR : Adjacent and Nearby alleles
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.
Wildtype, GG
Wildtype, CG
Wildtype, CC
Wildtype, GC
2. Perform LDR using allele-specific LDR primers and thermostable ligase. Allele specific oligonucleotides ligate to common 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

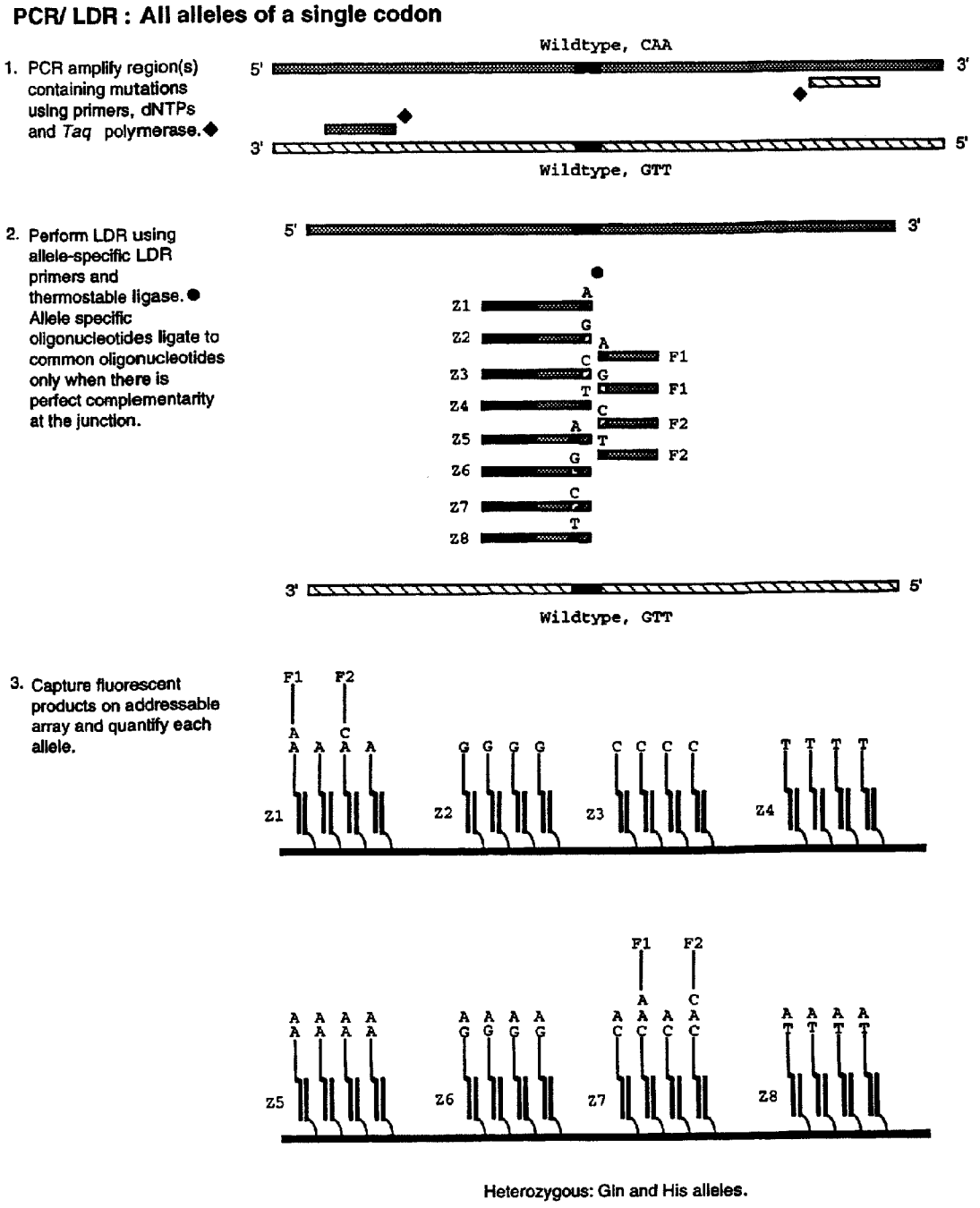

PCR/ LDR : All alleles of a single codon
1. PCR amplify region(s) containing mutations using primers, dNTPs and Taq polymerase.◆
Wildtype, CAA
5'
3'
3'
5'
Wildtype, GTT
2. Perform LDR using allele-specific LDR primers and thermostable ligase.● Allele specific oligonucleotides ligate to common oligonucleotides only when there is perfect complementarity at the junction.
Z1
Z2
Z3
Z4
Z5
Z6
Z7
Z8
F1
F2
Wildtype, GTT
3. Capture fluorescent products on addressable array and quantify each allele.
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

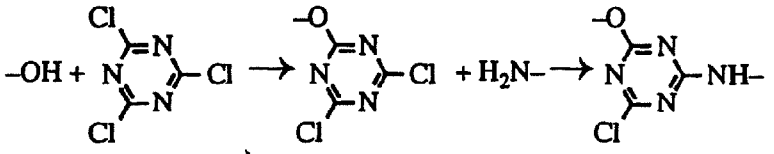
W = protecting group, e.g. Boc, Fmoc
Z = activating group, e.g. imidazole (Im), *p*-nitrophenol (OPnp), hydroxysuccinimide (OSu), pentafluorophenol (OPfp)
PEG = oligo or poly(ethylene glycol), backbone $(CH_2CH_2O)_n$ n = 6 to 200 (can also be grown by anionic polymerization with )
WSC = water soluble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



–OH + O=C=N– ⟶ –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 ⟶ $-OCH_2(C{=}O)H + H_2N- + NaCNBH_3 \longrightarrow -OCH_2CH_2NH-$

FIG. 11

FIG. 12A

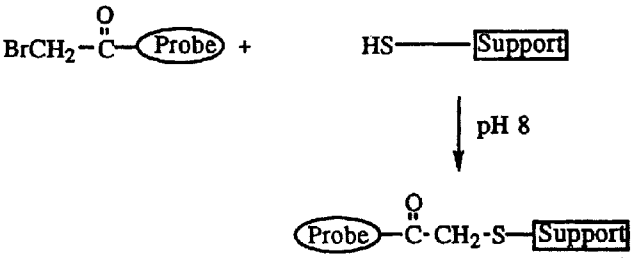


FIG. 12B

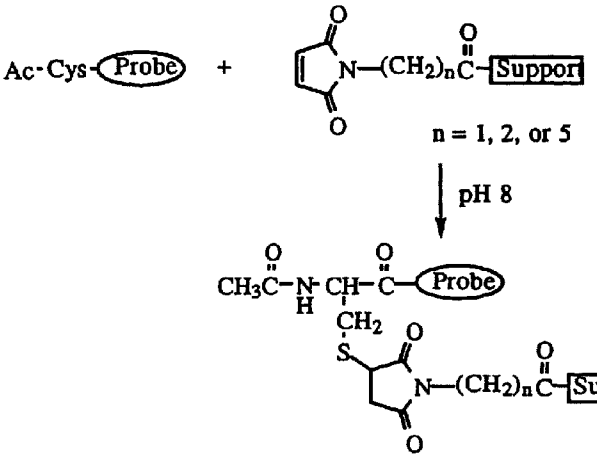
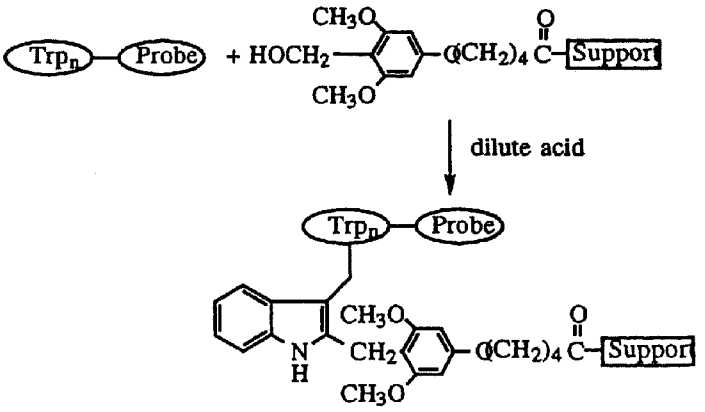


FIG. 12C



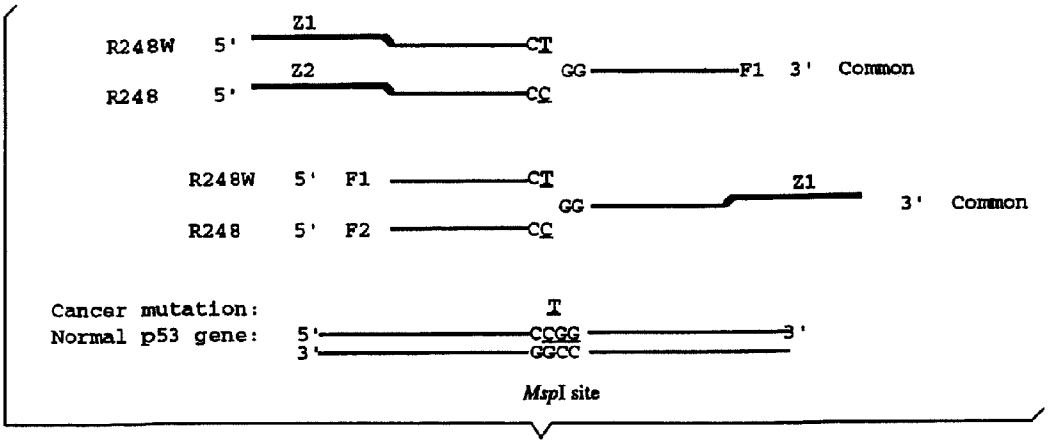


FIG. 13A

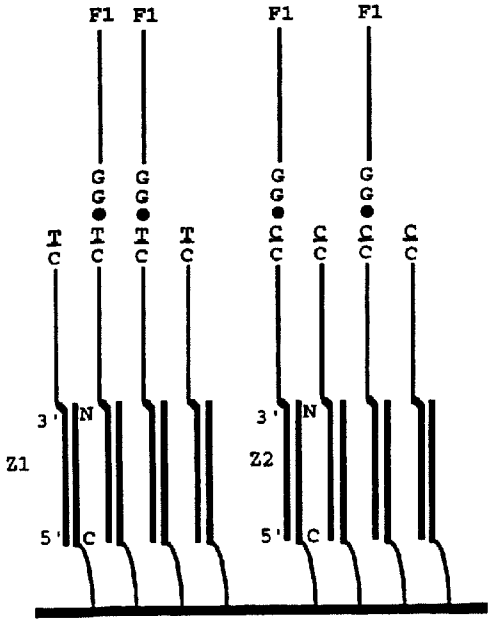


FIG. 13B

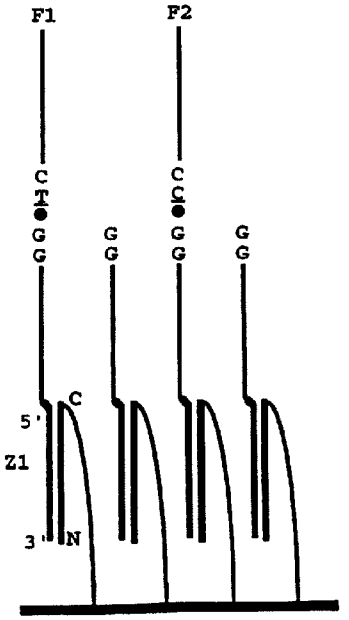


FIG. 13C

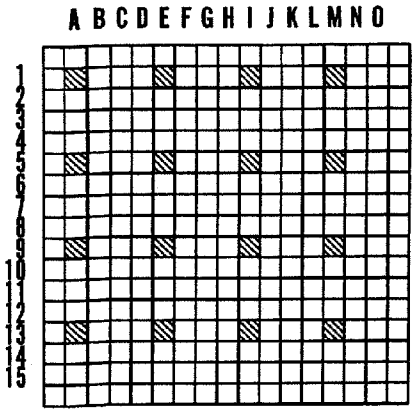


FIG. 14A

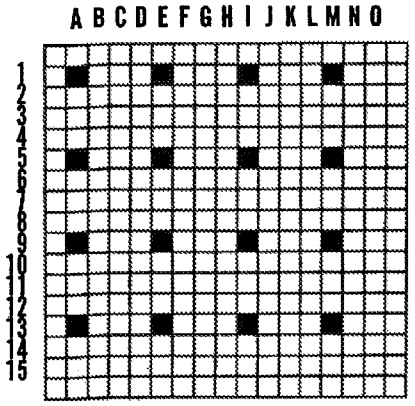


FIG. 14B

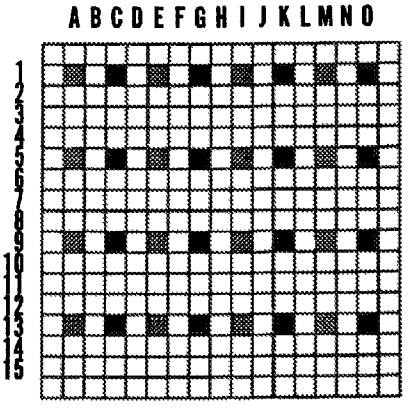


FIG. 14C

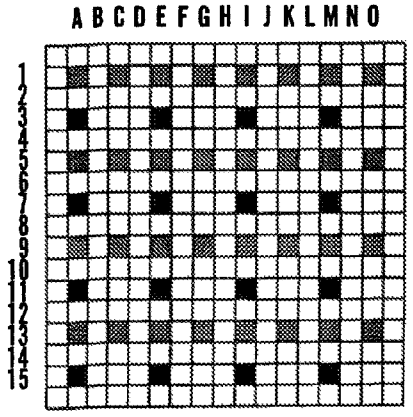


FIG. 14D

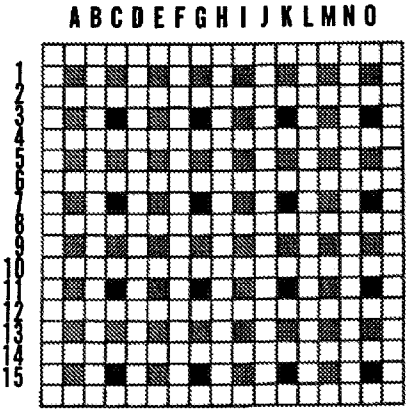


FIG. 14E

FIG. 15A

1st addition of unique 24mers.

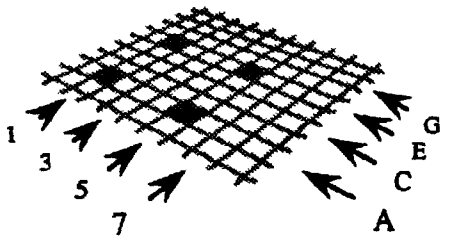


FIG. 15B

2nd addition of unique 24mers.

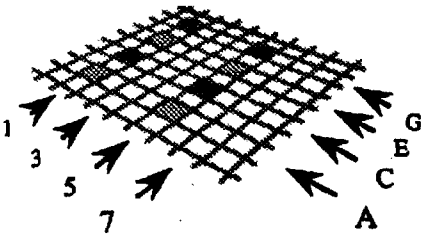


FIG. 15C

3rd addition of unique 24mers.

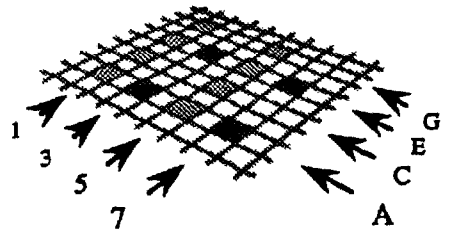
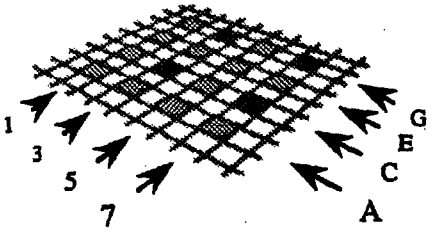


FIG. 15D

4th addition of unique 24mers.



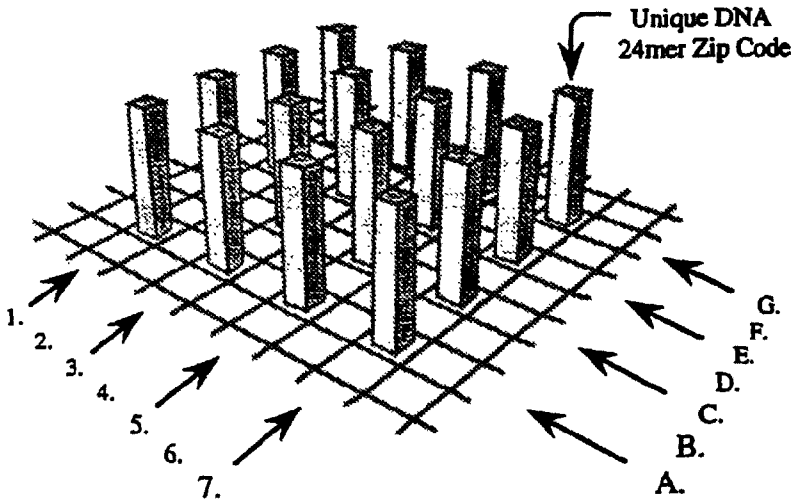


FIG. 15E

FIG. 16A

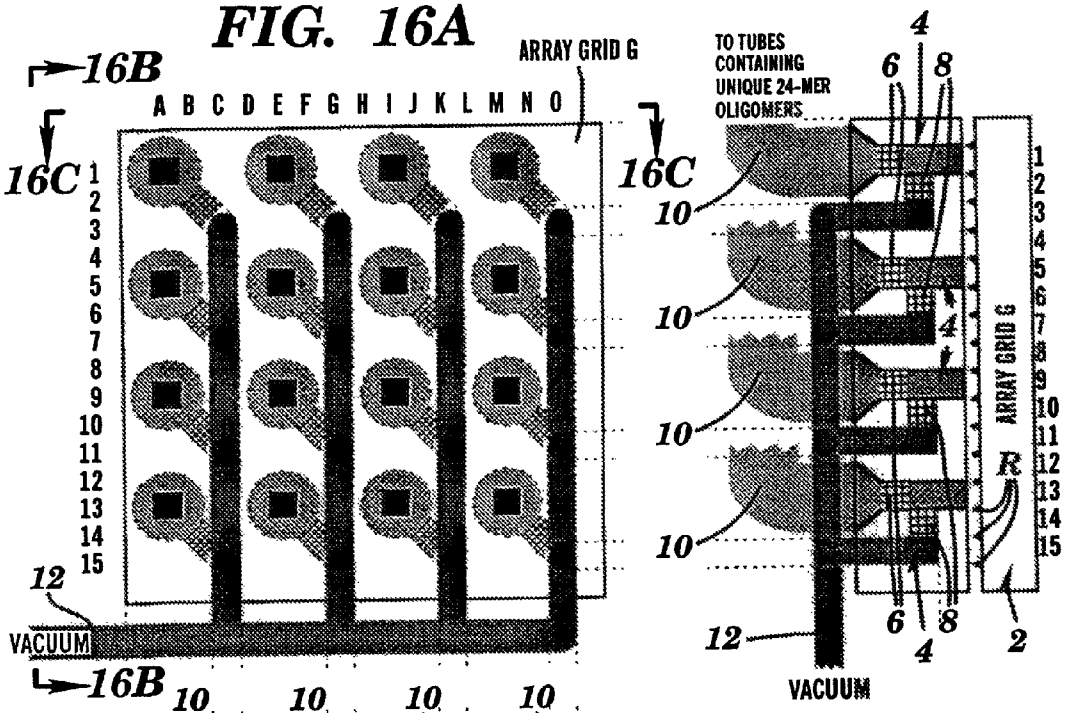


FIG. 16B

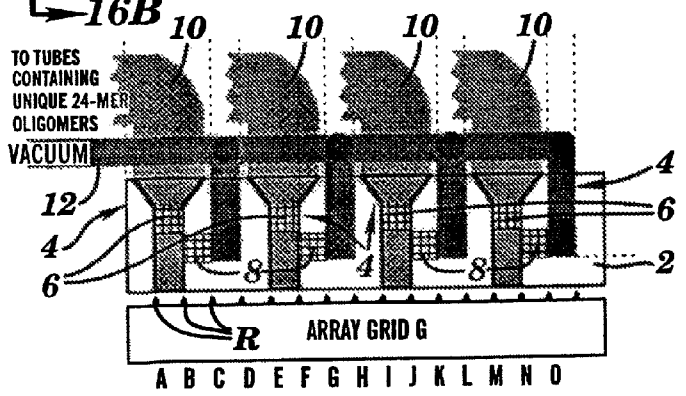


FIG. 16C

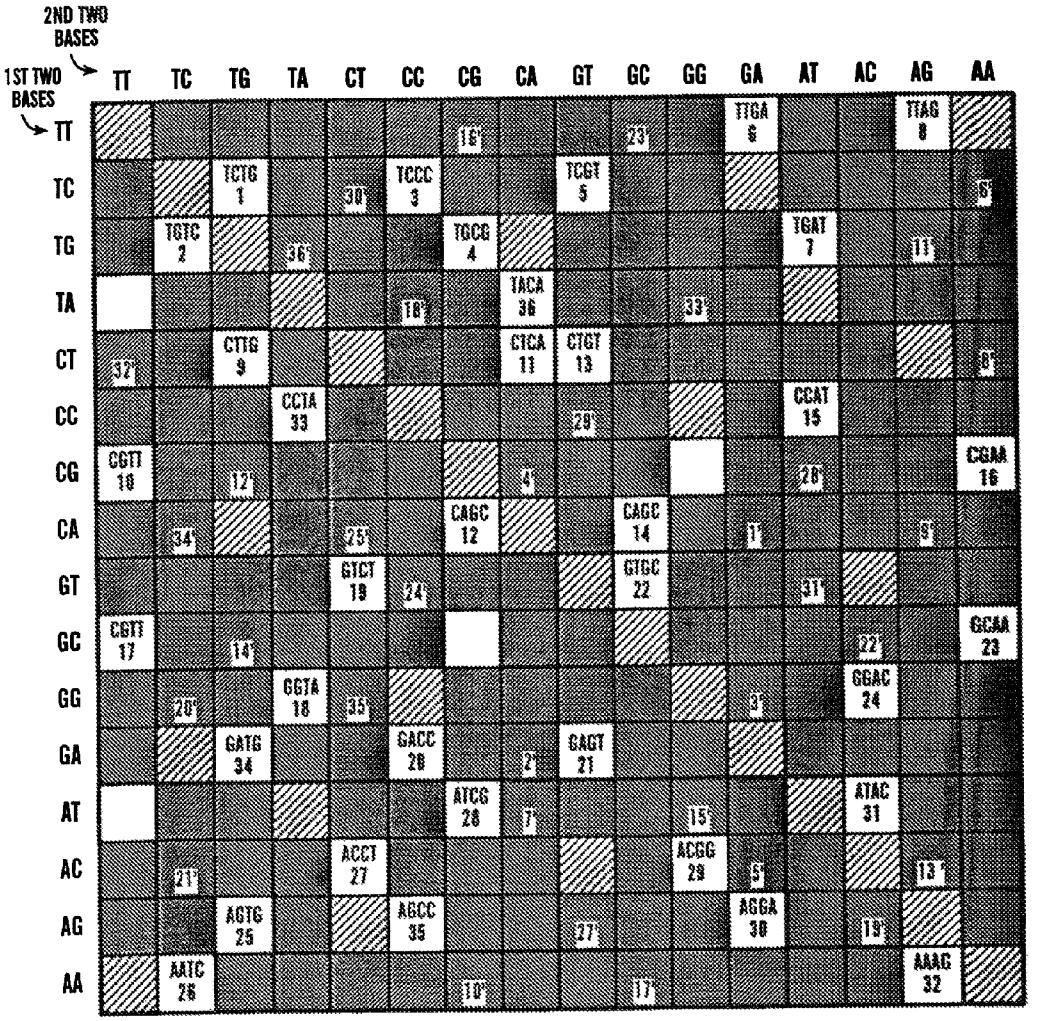

2ND TWO BASES
1ST TWO BASES
TT TC TG TA CT CC CG CA GT GC GG GA AT AC AG AA
TTGA 6
TTAG 8
TCTG 1
TCCC 3
TCGT 5
TGTC 2
TGCG 4
TGAT 7
TACA 36
CTTG 9
CTCA 11
CTGT 13
CCTA 33
CCAT 15
CGTT 18
CGAA 16
CAGC 12
CAGC 14
GTCT 19
GTGC 22
CGTT 17
GCAA 23
GGTA 18
GGAC 24
GATG 34
GACC 28
GAGT 21
ATCG 28
ATAC 31
ACCT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

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

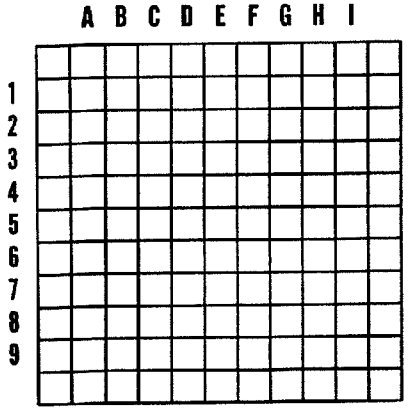


FIG. 19A

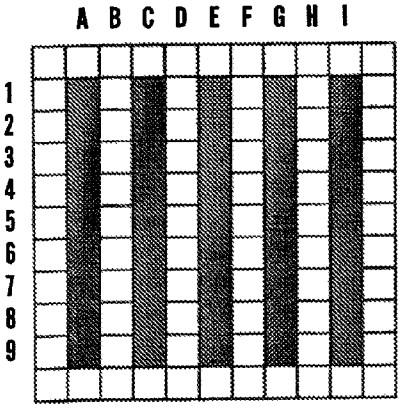


FIG. 19B

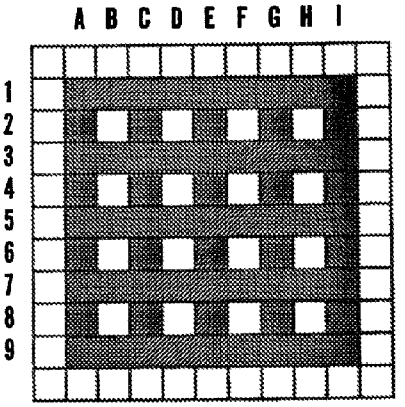


FIG. 19C

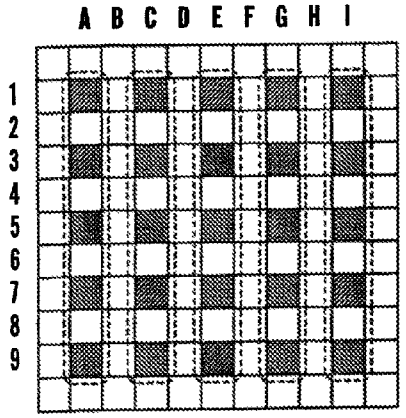


FIG. 19D

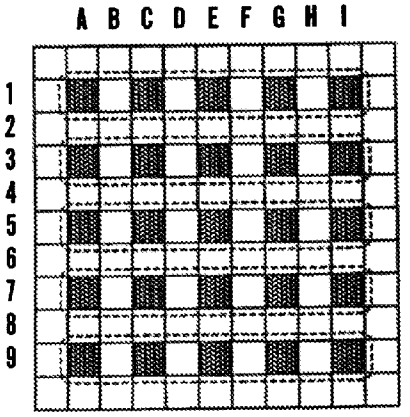


FIG. 19E

FIG. 20A

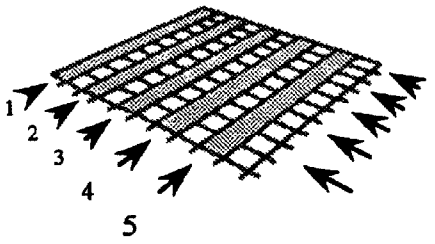
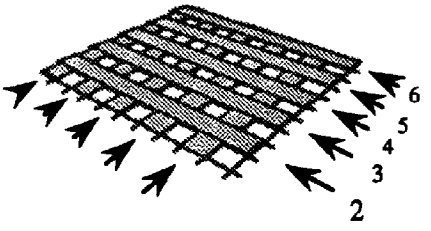


FIG. 20B



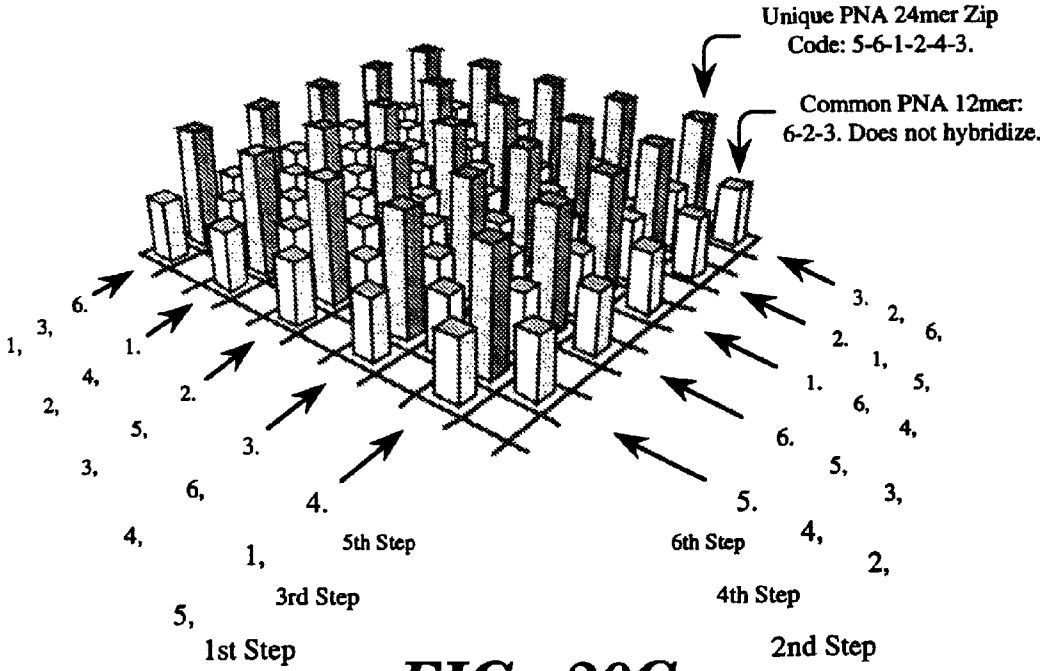


FIG. 20C

FIG. 21A



FIG. 21B



FIG. 21C

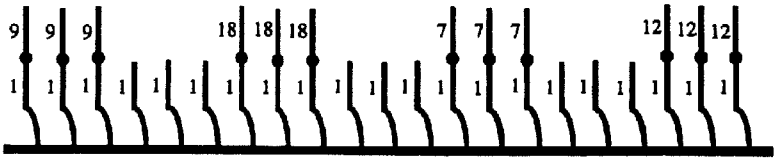


FIG. 21D

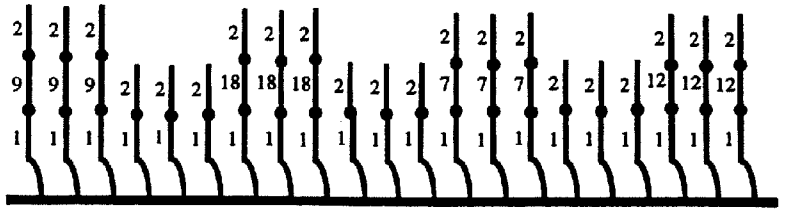


FIG. 21E

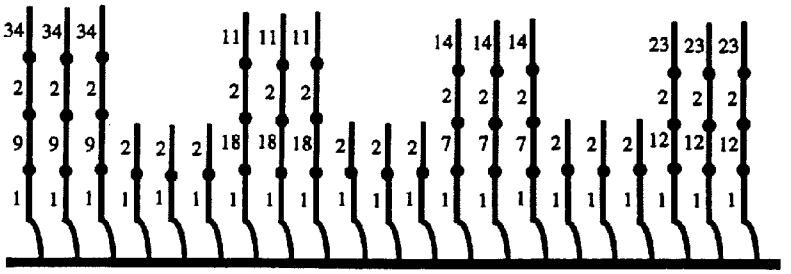


FIG. 21F

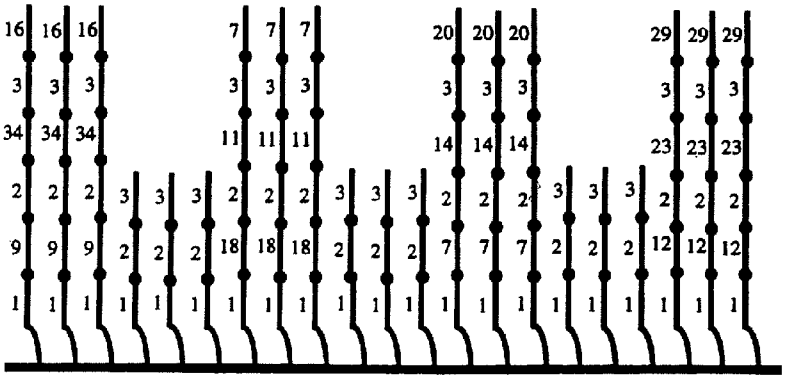


FIG. 22A

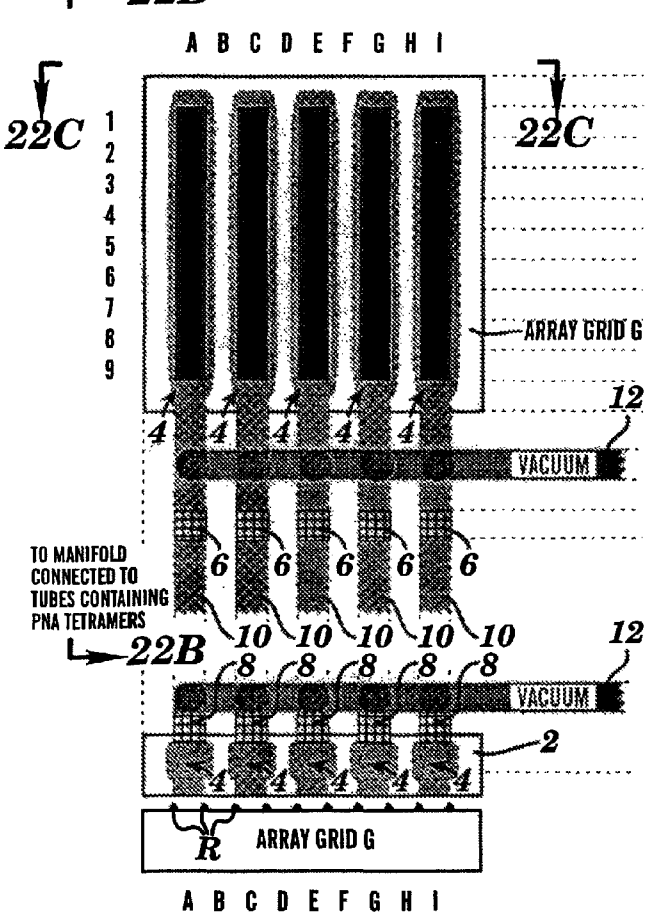


FIG. 22C

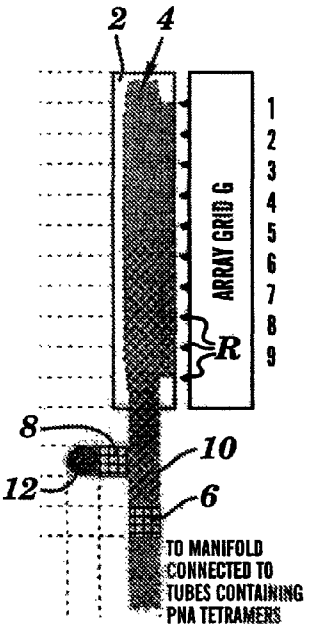


FIG. 22B

FIG. 23A

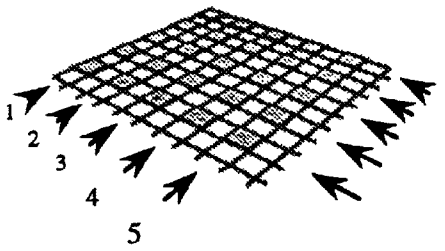
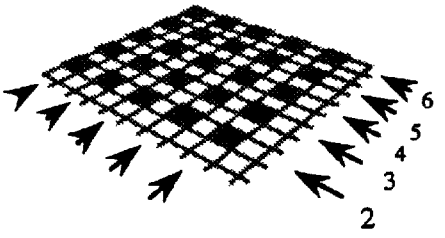


FIG. 23B



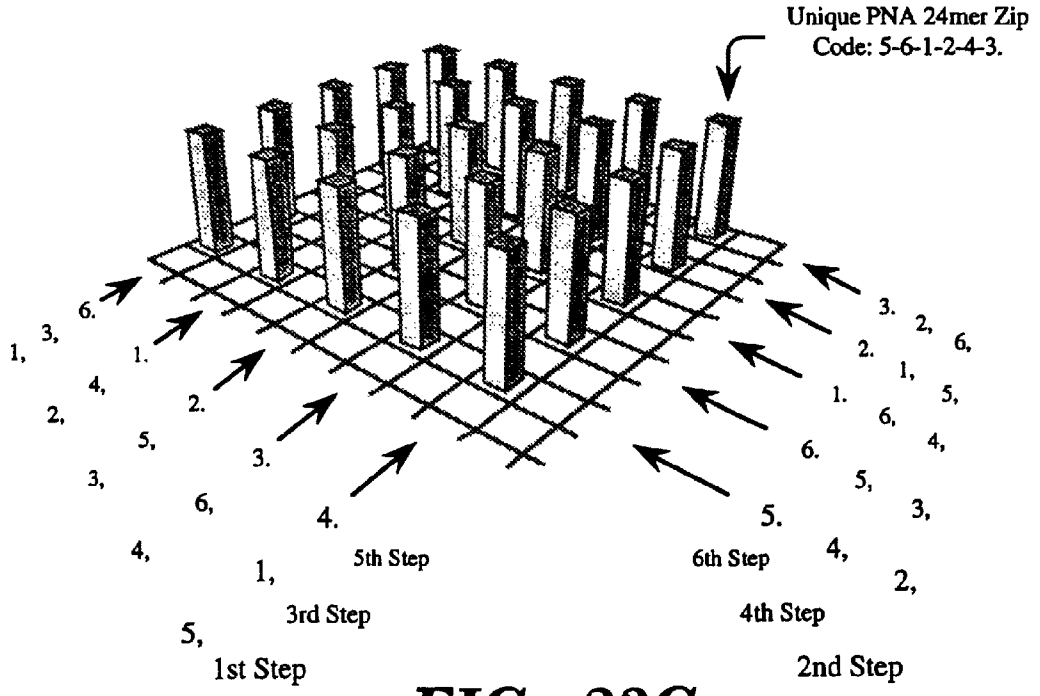


FIG. 23C

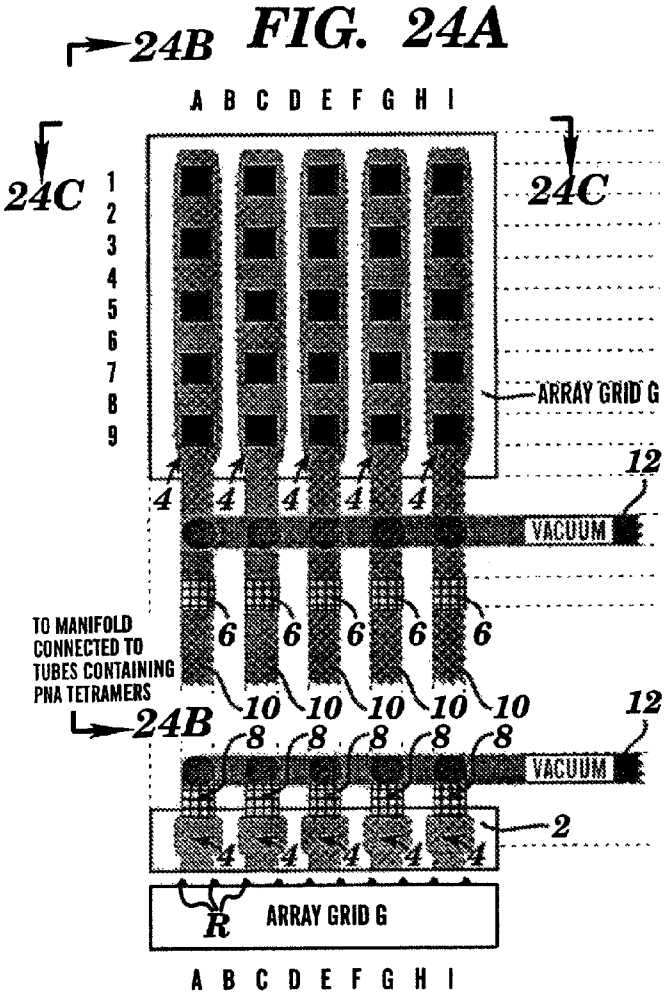


FIG. 24A

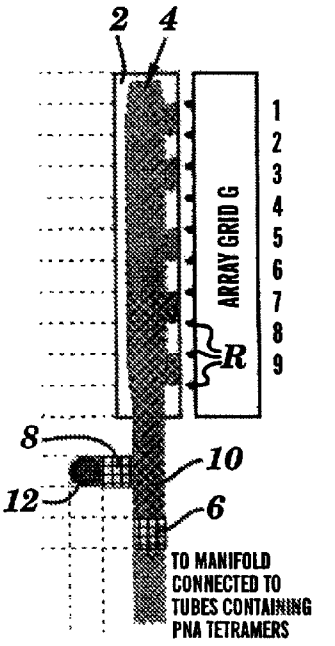


FIG. 24B

FIG. 24C

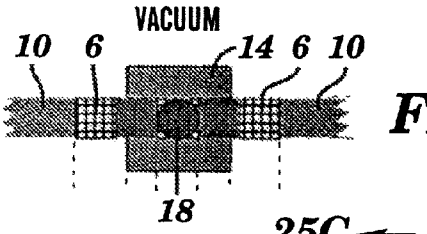


FIG. 25B

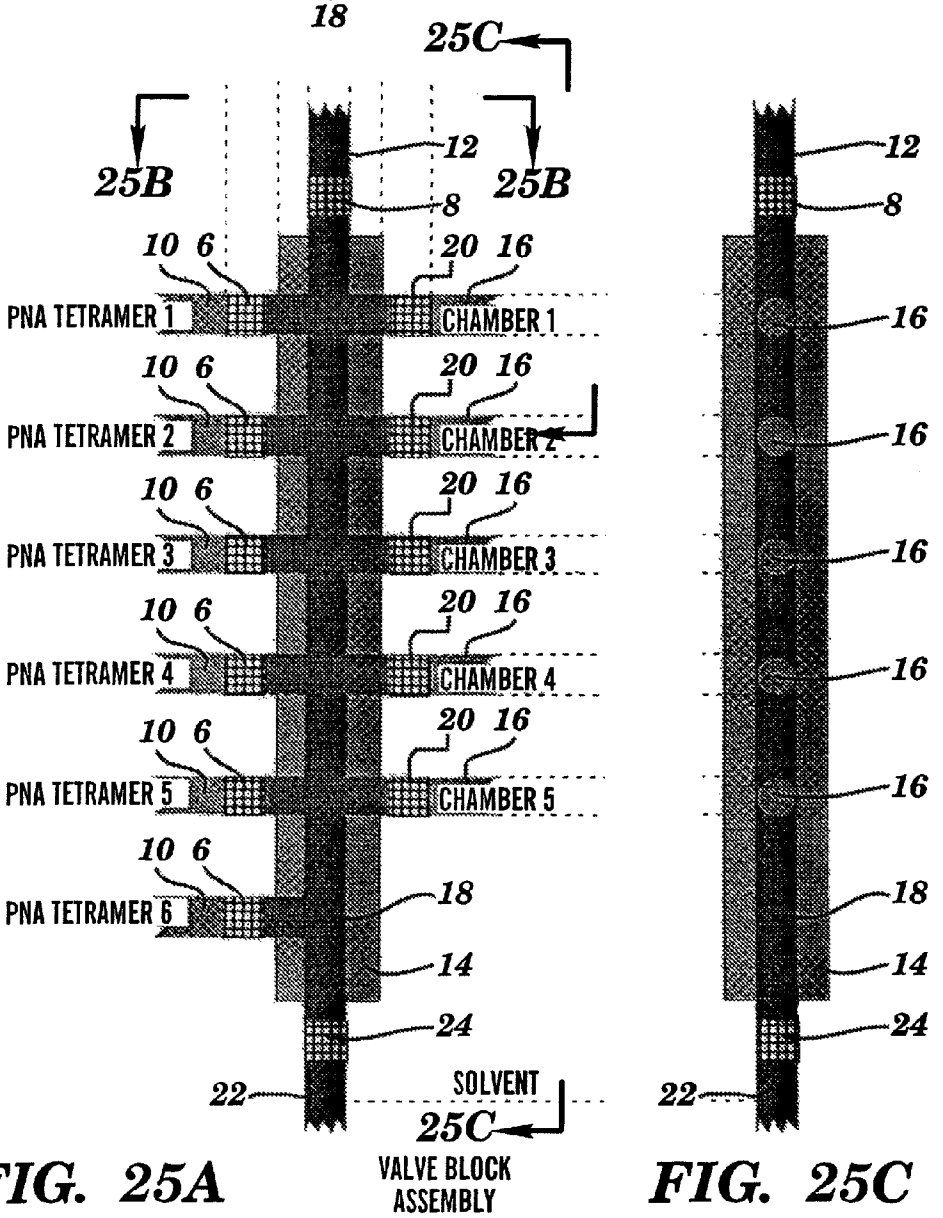


FIG. 25A

FIG. 25C

6 INPUTS AND 5 OUTPUTS

FIG. 26A

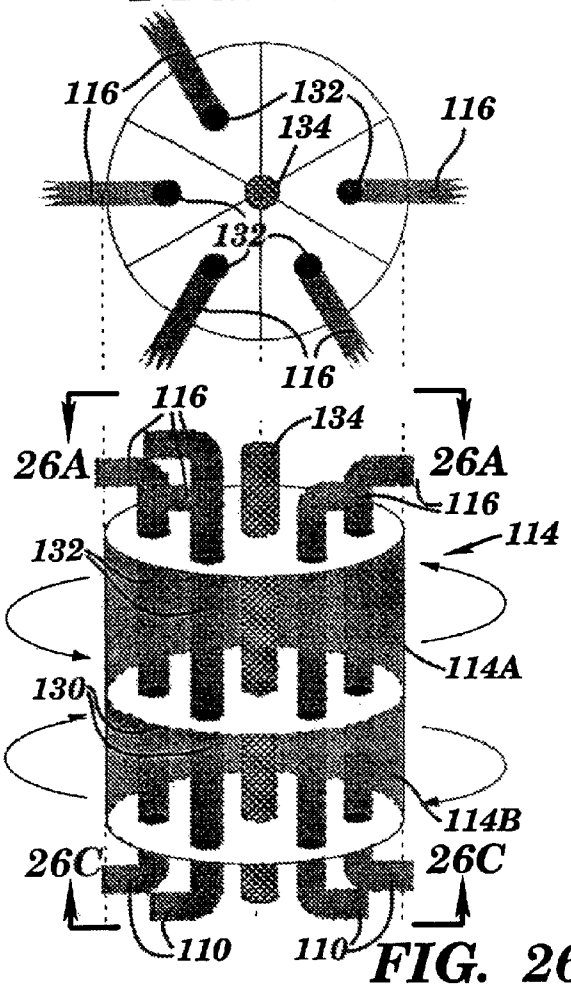


FIG. 26B

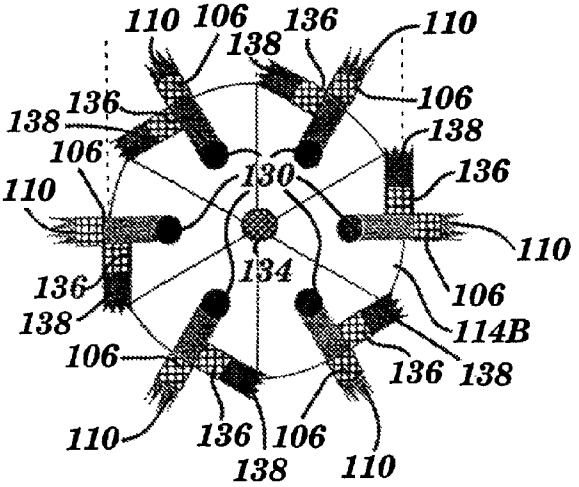


FIG. 26C

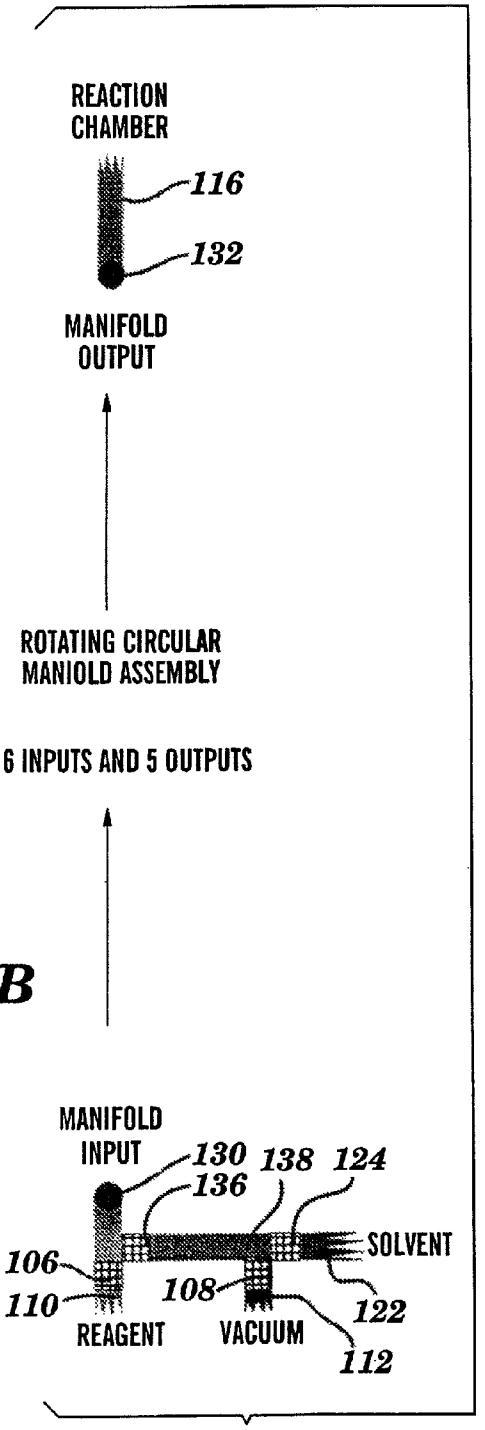


FIG. 26D

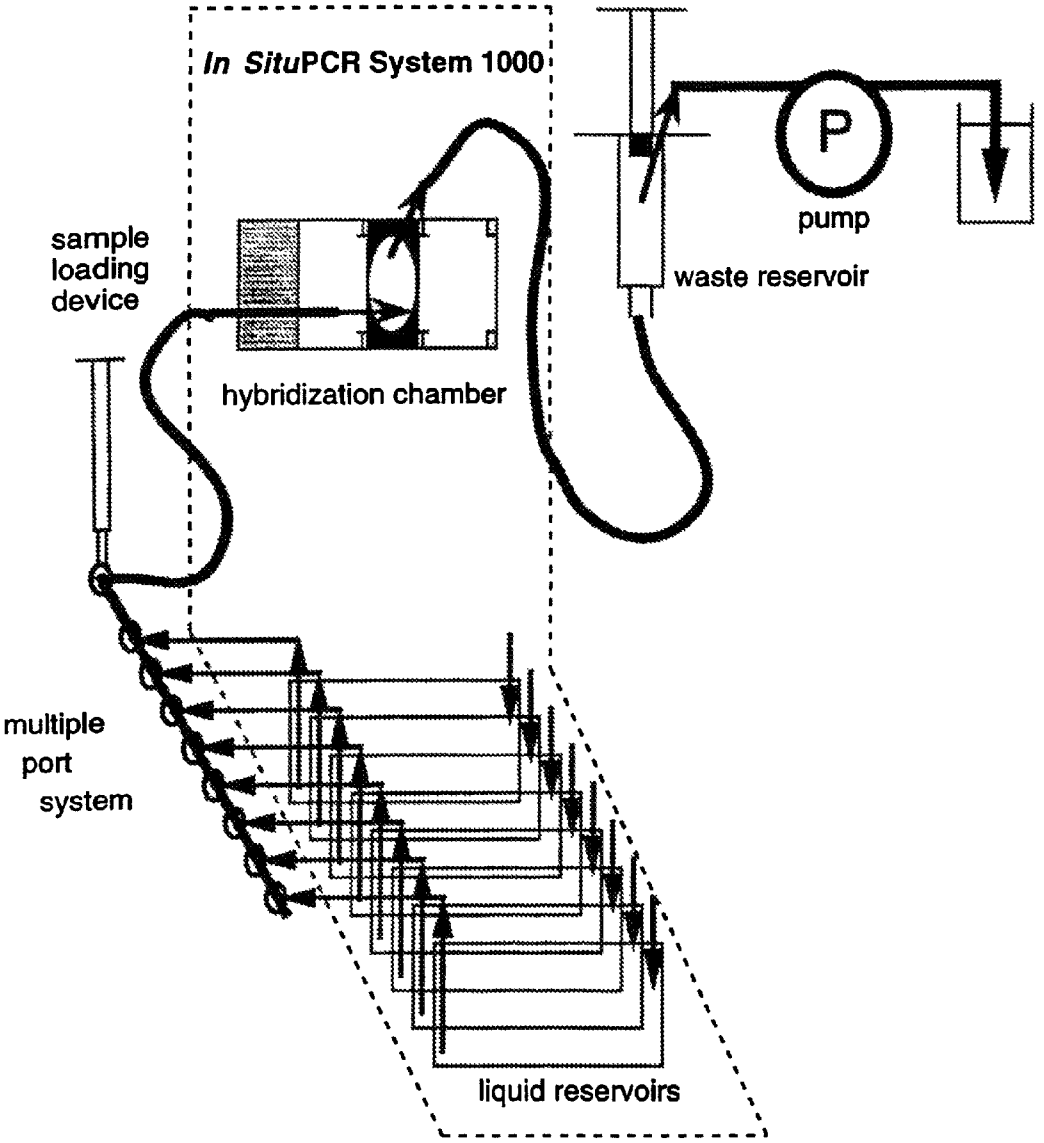

In SituPCR System 1000
P
pump
waste reservoir
sample loading device
hybridization chamber
multiple port system
liquid reservoirs

FIG. 27

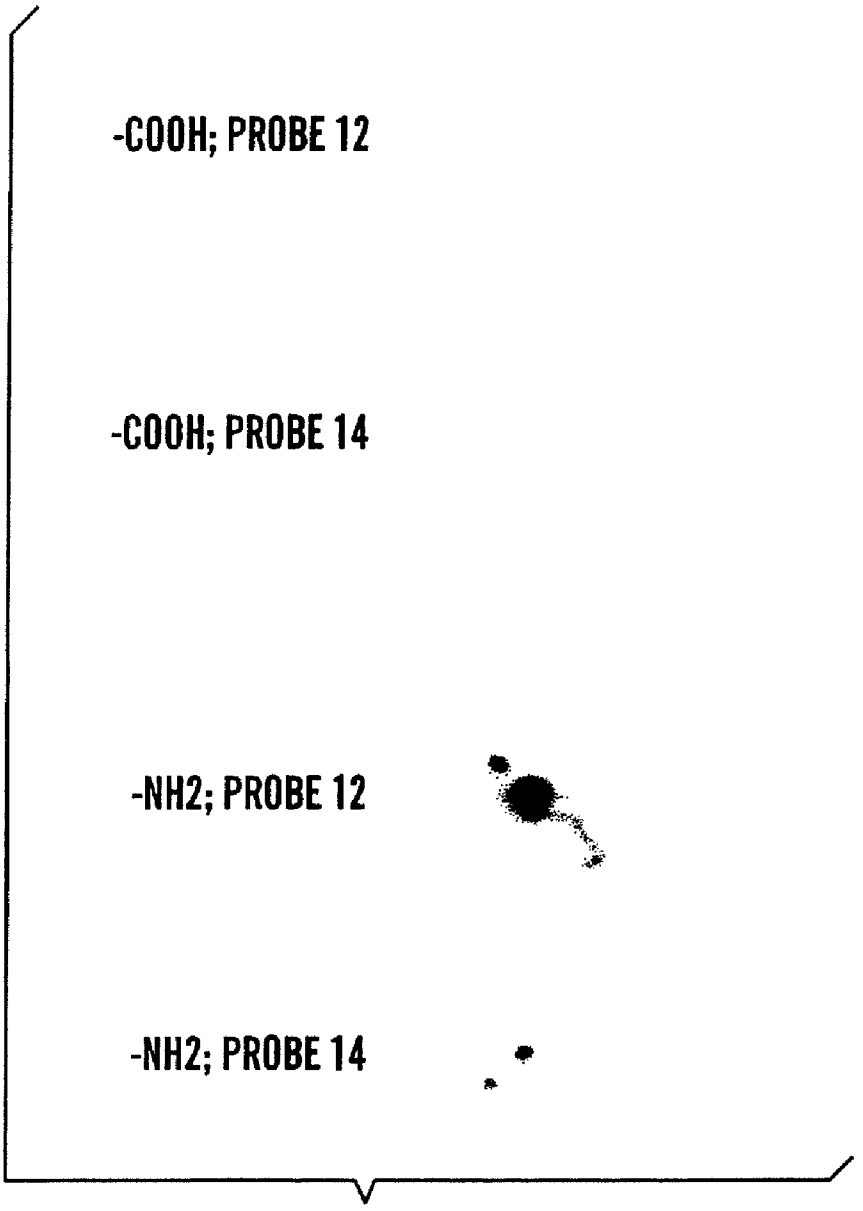


FIG. 28

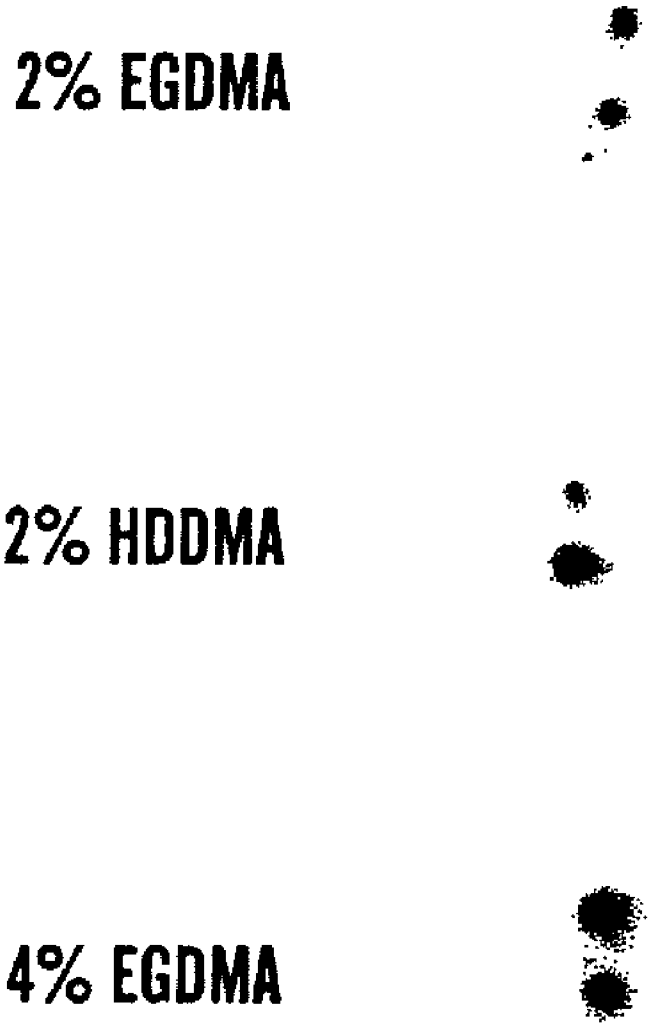


FIG. 29

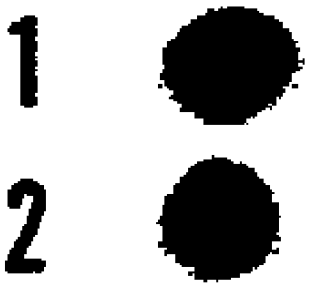


FIG. 30

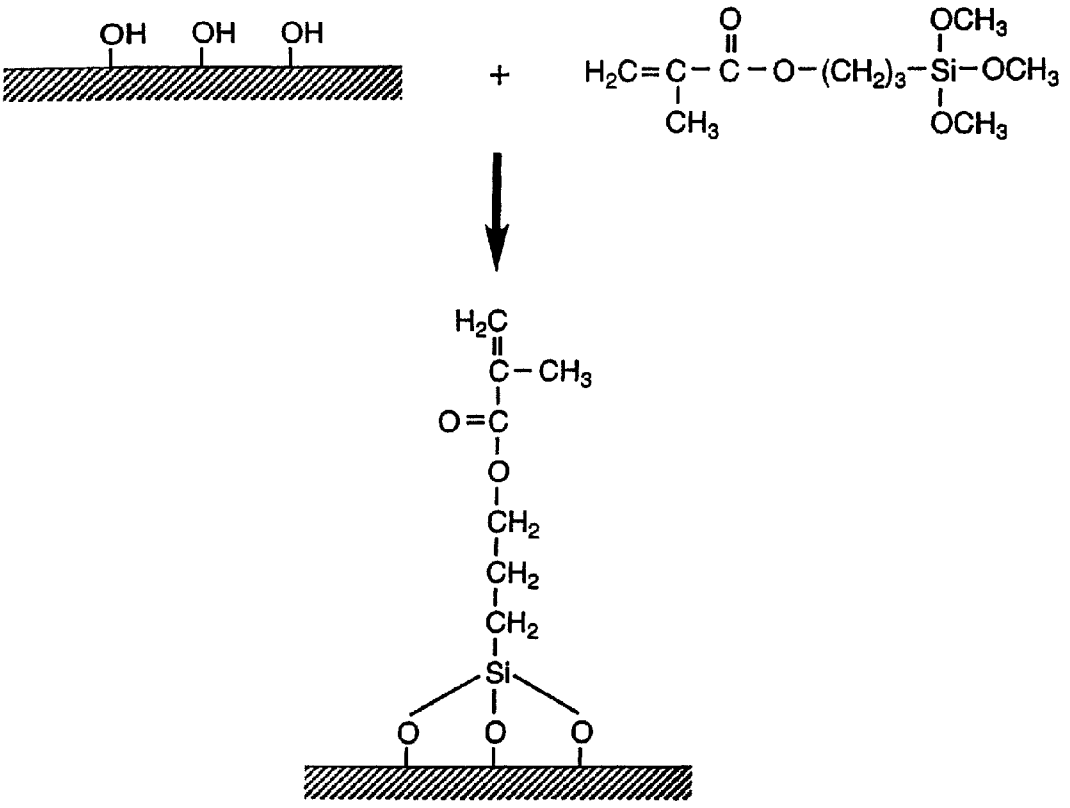

OH
OH
OH
+
O
OCH3
H2C=C-C-O-(CH2)3-Si-OCH3
CH3
OCH3
H2C
C-CH3
O=C
O
CH2
CH2
CH2
Si
O
O
O

FIG. 31

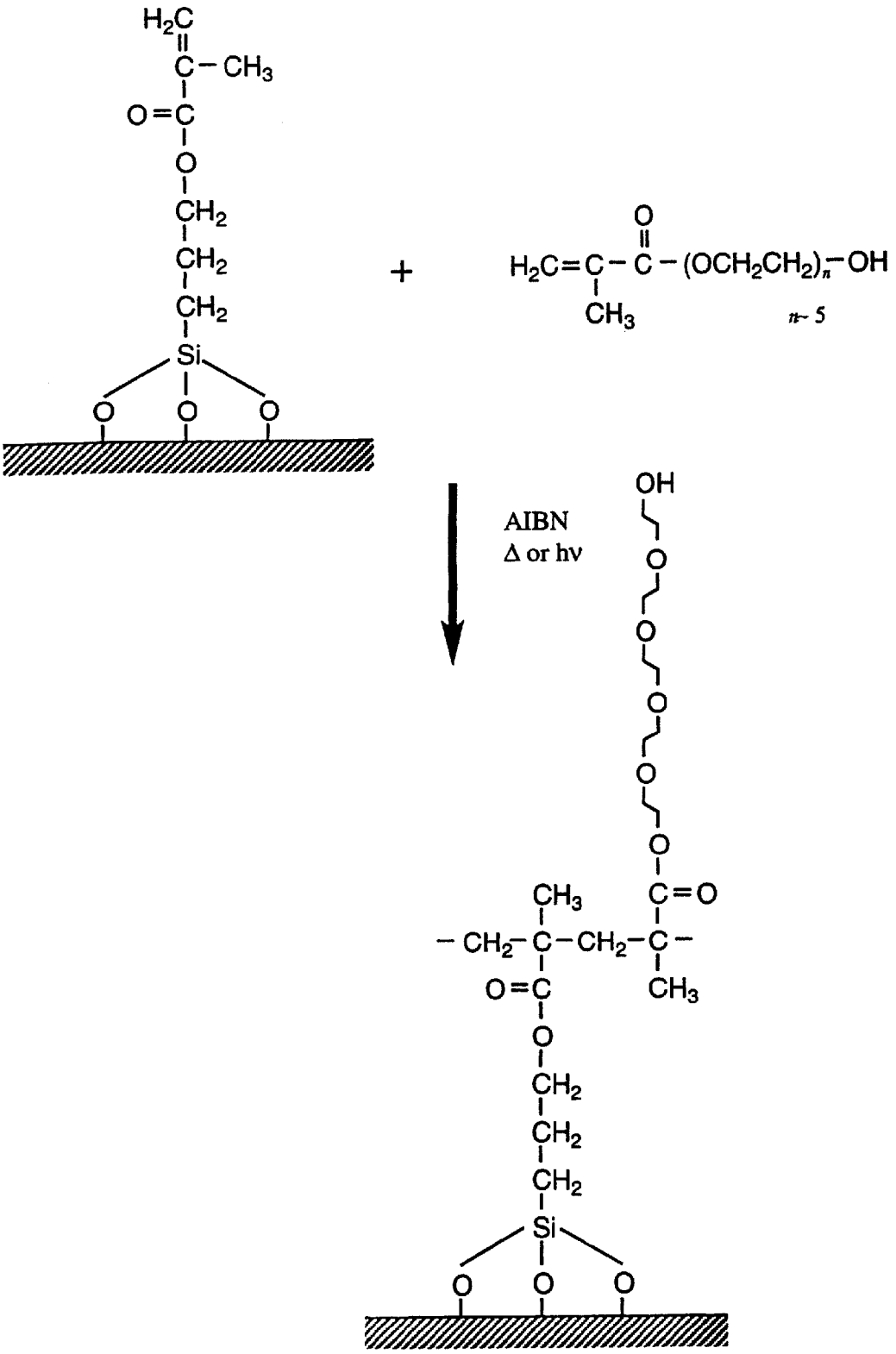


FIG. 32

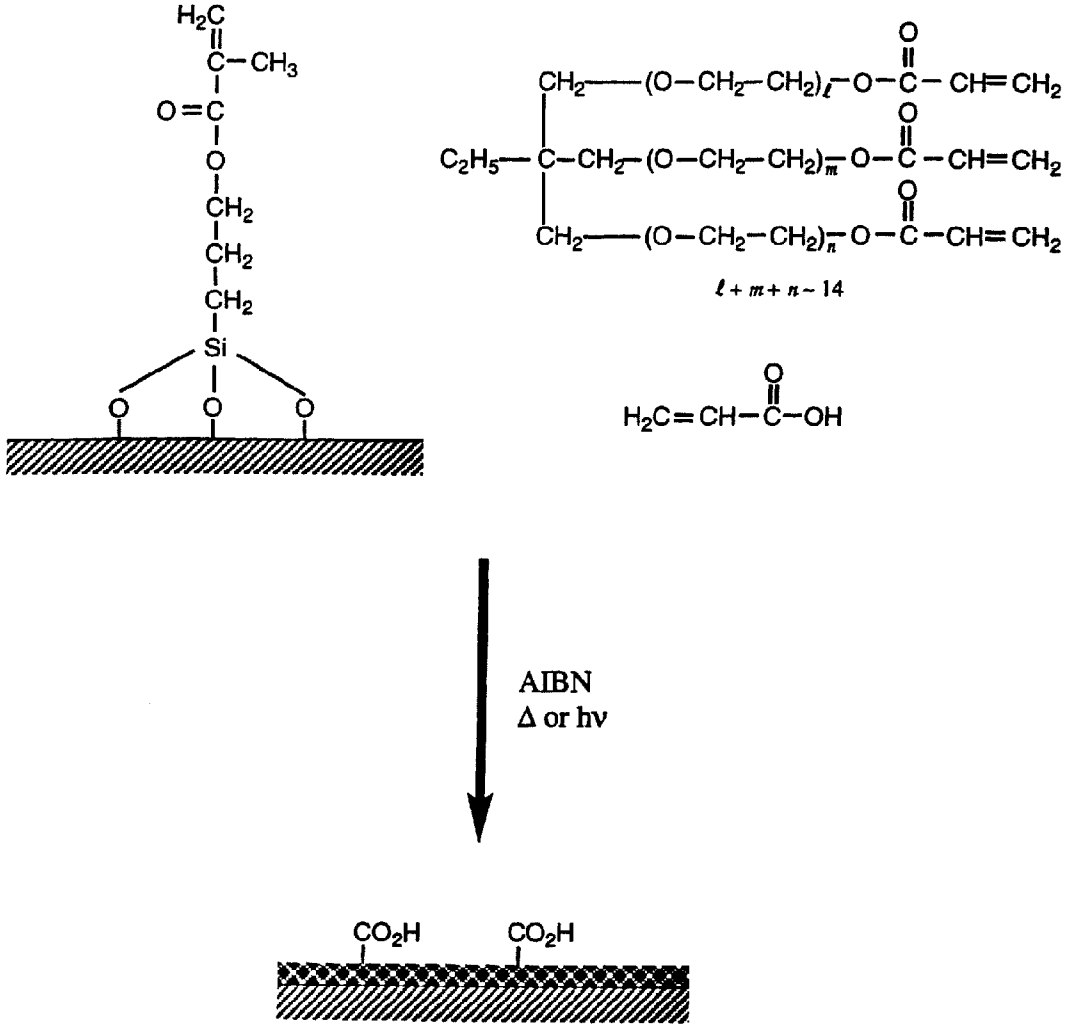


FIG. 33

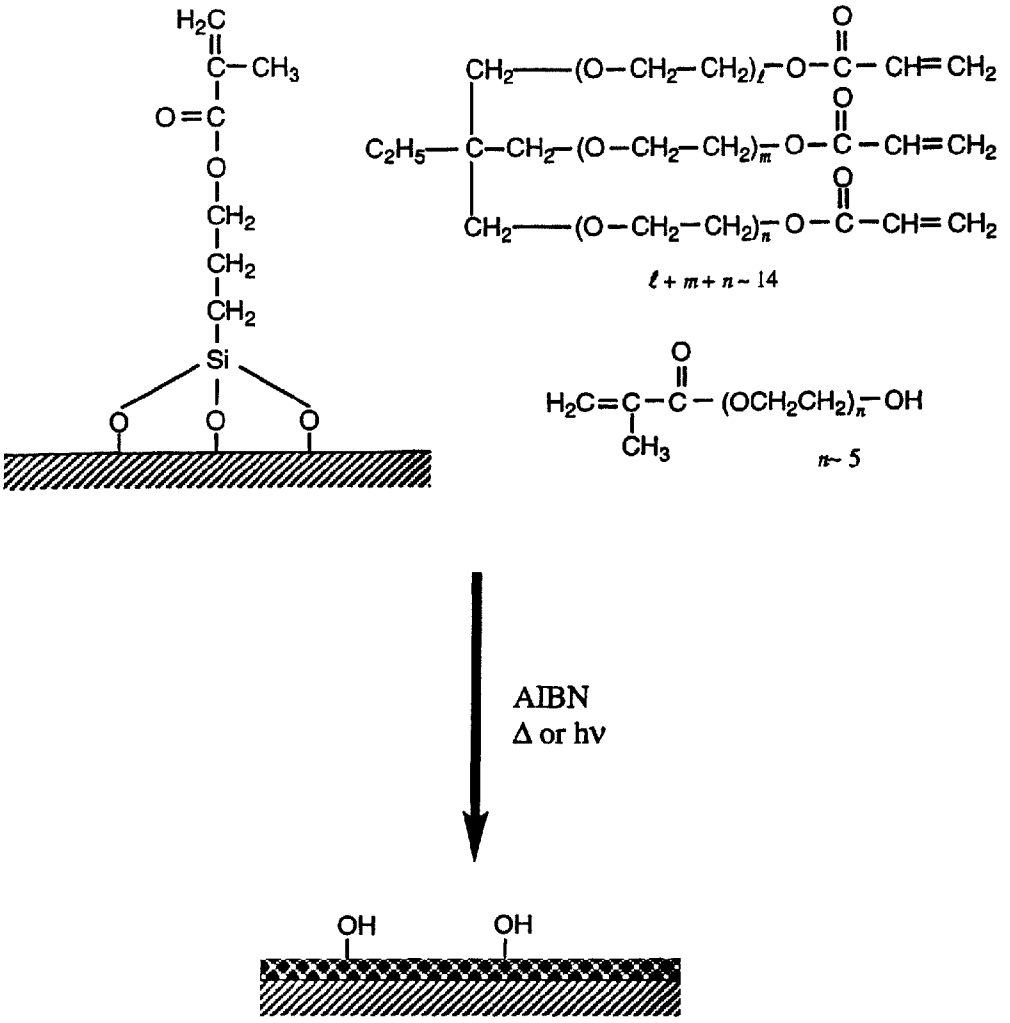


FIG. 34

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

The present application is a continuation of U.S. patent application Ser. No. 10/854,678, filed May 25, 2004, which is a continuation of U.S. patent application Ser. No. 08/794,851, filed Feb. 4, 1997, now U.S. Pat. No. 6,852,487, issued on Feb. 8, 2005, which claims the benefit of U.S. Provisional Patent Application Ser. No. 60/011,359, filed Feb. 9, 1996, which are hereby incorporated by reference in their entirety.

This invention was made with government support under grant number GM-41337-06 awarded by National Institutes of Health. The U.S. Government has certain rights in this invention.

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* 5:86-93 (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 polynucle-

otide. 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 FIGS. **13**A-C, 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**B, 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.

FIG. **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-G.

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-trimethoxy-silane.

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 transloca-

tions 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 **6***a*, 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 **6***b*, 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: 189-93 (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, yoghurt, 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 hybridiza-

tion would be in standard antiparallel 5' to 3' and 3' to 5'. Other modified sugar-phosphate backbones would be used in a similar fashion. FIG. **13**A 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). FIGS. **13**B-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.

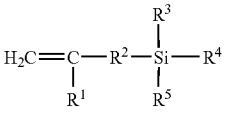
Surfaces on the solid substrate will usually, though not always, be composed of the same material as the substrate. Thus, the surface may be composed of any of a wide variety of materials, for example, polymers, plastics, ceramics, polysaccharides, silica or silica-based materials, carbon, metals, inorganic 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);

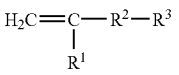
R^3 is an O-alkyl, alkyl, or halogen group;

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, COCl, 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.

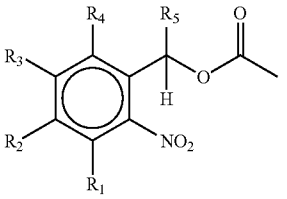
The linker molecules can be attached to the substrate via carbon-carbon bonds using, for example, (poly)tri-fluorochloroethylene surfaces or, preferably, by siloxane bonds (using, for example, glass or silicon oxide surfaces). Siloxane bonds with the surface of the substrate may be formed in one embodiment 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 oligo-tryptophanyl 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,* 13:1008-1017 (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).

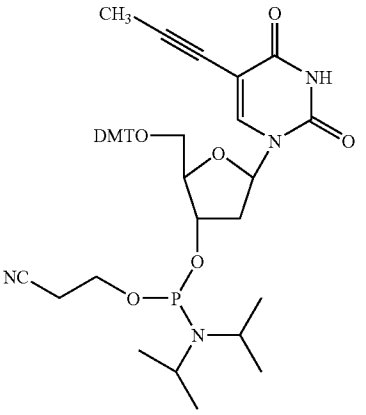
Although considerable progress has been made in constructing oligonucleotide arrays, some containing as many as 256 independent addresses, these procedures are less preferred, for detecting specific DNA sequences by hybridizations. More particularly, arrays containing longer oligonucleotides can currently 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.

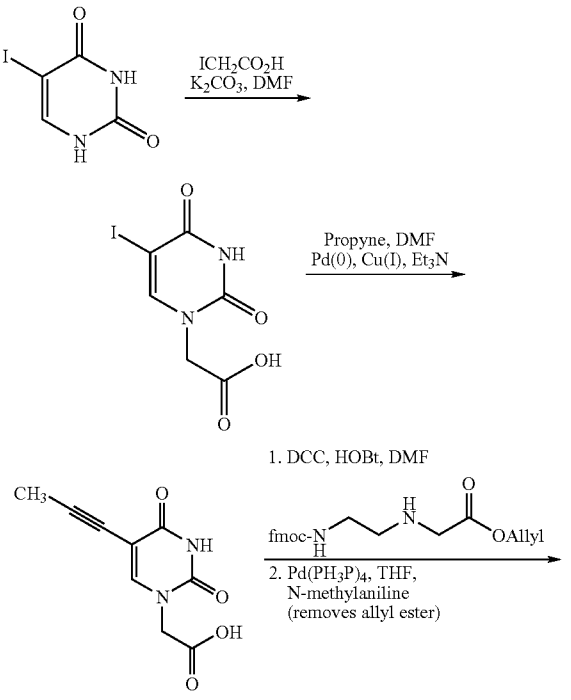
It is desirable to produce a solid support with a good loading of oligonucleotide or PNA oligomer in a relatively small, but well-defined area. Current, commercially available fluorescent imagers can detect a signal as low as 3 attomoles per 50 μm square pixel. Thus, a reasonable size address or "spot" 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 nonfluorescent, 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.

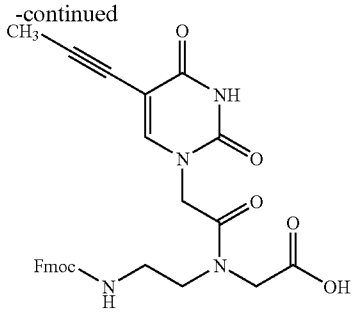
The melting temperature of DNA/DNA duplexes can be estimated as [4n(G•C)+2m(A•T)]° C. Oligonucleotide capture can be optimized by narrowing the T_m difference between duplexes formed by capture oligonucleotides and the complementary addressable array-specific portions hybridized to one another resulting 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):



-continued



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 A1, E1, I1, M1, A5, E5, IS, M5, A9, E9, I9, M9, A13, E13, I13, and M13 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 A1, E1, A5, and E5. Next, as shown in FIG. **15**B, the machine is shifted horizontally and attaches 4 oligomers at locations C1, G1, C5, and G5. Next, as shown in FIG. **15**C, the machine is repositioned and attaches 4 oligomers at locations A3, E3, A7, and E7. Finally, as shown in FIG. **15**D, the machine attaches the 4 remaining oligomers at positions C3, G3, C7, and G7. 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

TABLE 1-continued

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
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 NH2 → COOH)	G + C
Zip 11	1-4-3-6-6-1	TGCG-GGTA-CAGC-ACCT-ACCT-TGCG (SEQ ID NO: 2)	15
Zip 12	2-4-4-6-1-1	ATCG-GGTA-GGTA-ACCT-TGCG-TGCG (SEQ ID NO: 3)	14
Zip 13	3-4-5-6-2-1	CAGC-GGTA-GACC-ACCT-ATCG-TGCG (SEQ ID NO: 4)	15
Zip 14	4-4-6-6-3-1	GGTA-GGTA-ACCT-ACCT-CAGC-TGCG (SEQ ID NO: 5)	14
Zip 15	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 FIG. **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
5'-ATCG GGTA GGTA ACCT TGCG TGCG-3'  (SEQ ID NO: 7)

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

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 24-mer 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-G). 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 third round synthesis of PNA rows. The first row contains tetramer 3. FIG. **21**F shows the structure of the array after third round synthesis of columns, adding tetramers 16, 7, 20, 29. Note that all 24-mer 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 at 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 (FIG. **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-**24**B and line **24**C-**24**C 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 (FIG. **25**A-C) will route each tetramer to the appropriate row. Alternatively, a cylindrical manifold (FIG. **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**. FIG. **25**A is a top view of valve block assembly **14**, while side views FIGS. **25**B and **25**C are, respectively, taken along line **25**B-**25**B and line **25**C-**25**C of FIG. **25**A. 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 Enzymol., 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 non-specifically 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 21G 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 21 G 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 oligonucleotide 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.

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)

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 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

TABLE 5-continued

Quantification of captured oligonucleotide probes

Crosslinker	Percentage crosslinker	probe 12 (pic)*	probe 12 (amol)
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 mismatches, 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 M1,1'-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 nmol/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 1.5 ml 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

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	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

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	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

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	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. Jönsson, 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 glove-box 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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What is claimed:

1. A collection of capture oligonucleotides, wherein each type of capture oligonucleotide of the collection has greater than sixteen nucleotides and comprises a nucleotide sequence that differs from the nucleotide sequence of other types of capture oligonucleotides in the collection by at least 25% when aligned, and wherein each capture oligonucleotide of the collection hybridizes to a nucleic acid molecule comprising a complementary nucleotide sequence under uniform hybridization conditions.

2. The collection of claim **1**, wherein each capture oligonucleotide in the collection comprises 20-25 mers.

3. The collection of claim **1**, wherein one or more of the capture oligonucleotides in the collection is hybridized to a nucleic acid molecule comprising a complementary nucleotide sequence.

4. A group of beads, wherein each bead of the group comprises one or more types of capture oligonucleotides from a collection of capture oligonucleotides, said capture oligonucleotides comprising greater than sixteen nucleotides with each type of capture oligonucleotide of the collection comprising a nucleotide sequence that differs from the nucleotide sequence of other types of capture oligonucleotides of the collection by at least 25% when aligned, and wherein each capture oligonucleotide in the collection hybridizes to a nucleic acid molecule comprising a complementary nucleotide sequence under uniform hybridization conditions.

5. The group of beads of claim **4**, wherein each capture oligonucleotide of the collection has a length of 20-25 mers.

6. The group of beads of claim **4**, wherein one or more of the capture oligonucleotides of the collection is hybridized to a nucleic acid molecule comprising a complementary nucleotide sequence.

7. A device comprising:

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

an array of capture oligonucleotides on the solid support at the array of positions, wherein each type of capture oligonucleotide on the solid support comprises a nucleotide sequence that is greater than sixteen nucleotides with a nucleotide sequence that differs from the nucle-

otide sequence of other types of capture oligonucleotides on the solid support by at least 25% when aligned; and

one or more target nucleic acid molecules hybridized to complementary portions of the capture oligonucleotides on the solid support, wherein the capture oligonucleotides hybridize to the complementary portions of the target nucleic acid molecules under uniform hybridization conditions.

8. The device according to claim **7**, each capture oligonucleotide has a length of 20-25 mers.

9. The device according to claim **7**, wherein different capture oligonucleotides are attached at different array positions on the solid support.

10. The device according to claim **7**, 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.

11. The device according to claim **7**, wherein the solid support is in a form selected from the group consisting of slides, discs, membranes, films, and composites thereof.

12. The device according to claim **7**, wherein the solid support has an array of positions with capture oligonucleotide attached to all of the array of positions.

13. The device according to claim **7**, wherein the solid support has wells, raised regions, or etched trenches.

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

15. The device according to claim **7**, wherein a linker couples the capture oligonucleotides to the solid support.

16. The device according to claim **15**, wherein the linker comprises a silane on a surface of the solid support.

17. The device according to claim **7**, wherein the solid support is functionalized with olefin, amino, hydroxyl, silanol, aldehyde, keto, halo, acyl halide, or carboxyl groups.

18. The device according to claim **17**, 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.

19. The device according to claim **17**, wherein the solid support is functionalized with an olefin-containing silane.

20. The device according to claim **19**, 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.

21. The device according to claim **19**, wherein the solid support is polymerized with an olefin containing monomer.

22. The device according to claim **21**, wherein the olefin-containing monomer contains a functional group.

23. The device according to claim **22**, 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.

24. The device according to claim **21**, 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, trimethylolpropane ethoxylate (7/3 EO/OH) triacrylate, trimethylolpropane propoxylate (1 PO/OH) triacrylate, trimethylolpropane propoxylate (2 PO/OH) triacrylate, and mixtures thereof.

25. The device according to claim **7**, wherein the linker or support is non-hydrolyzable.

26. The device according to claim **7**, wherein the array is reusable for repeatedly hybridizing oligonucleotides to the array of capture oligonucleotides on the solid support.

27. The device according to claim **7**, wherein each of the capture oligonucleotides on the solid support has a different nucleotide sequence.

* * * * *