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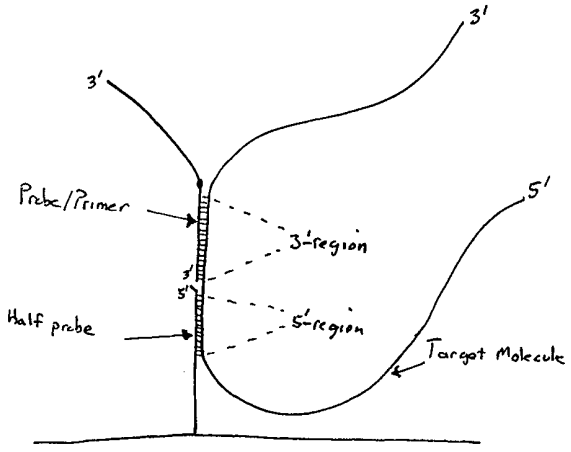

(57) Abstract

Disclosed are compositions and a method for detecting single nucleic acid molecules using rolling circle amplification (RCA) of single–stranded circular templates, referred to as amplification target circles, primed by immobilized primers. In one form of the method, referred to as a bipartite primer rolling circle amplification, (BP–RCA), RCA of the amplification target circle (ATC) depends on the formation of a primer by target–mediated ligation. In the presence of a nucleic acid molecule having the target sequence, a probe and a combination probe/primer oligonucleotide can hybridize to adjacent sites on the target sequence allowing the probes to be ligated together. By attaching the first probe to a substrate such as a bead or glass slide, unligated probe/primer can be removed after ligation. The only primers remaining will be primers ligated, via the probe portion of the probe/primer, to the first probe. The ligated primer can then be used to prime replication of its cognate ATC. In this way, an ATC will only be replicated if the target sequence (to which its cognate probe/primer is complementary) is present. BP–RCA is useful, for example, for determining which target sequences are present in a nucleic acid sample, or for determining which samples contain a target sequence.

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METHOD FOR DETECTING NUCLEIC ACIDS USING TARGET-MEDIATED LIGATION OF BIPARTITE PRIMERS

The disclosed invention is generally in the field of assays for detection of nucleic acids, and specifically in the field of nucleic acid amplification and mutation detection.

A number of methods have been developed which permit the implementation of extremely sensitive diagnostic assays based on nucleic acid detection. Most of these methods employ exponential amplification of targets or probes. These include the polymerase chain reaction (PCR), ligase chain reaction (LCR), self-sustained sequence replication (3SR), nucleic acid sequence based amplification (NASBA), strand displacement amplification (SDA), and amplification with Qβ replicase (Birkenmeyer and Mushahwar, *J. Virological Methods*, **35**:117-126 (1991); Landegren, *Trends Genetics*, **9**:199-202 (1993)).

While all of these methods offer good sensitivity, with a practical limit of detection of about 100 target molecules, all of them suffer from relatively low precision in quantitative measurements. This lack of precision manifests itself most dramatically when the diagnostic assay is implemented in multiplex format, that is, in a format designed for the simultaneous detection of several different target sequences.

In practical diagnostic applications it is desirable to assay for many targets simultaneously. Such multiplex assays are typically used to detect five or more targets. It is also desirable to obtain accurate quantitative data for the targets in these assays. For example, it has been demonstrated that viremia can be correlated with disease status for viruses such as HIV-1 and hepatitis C (Lefrere *et al.*, *Br. J. Haematol.*, **82**(2):467-471 (1992), Gunji *et al.*, *Int. J. Cancer*, **52**(5):726-730 (1992), Hagiwara *et al.*, *Hepatology*, **17**(4):545-550 (1993), Lu *et al.*, *J. Infect. Dis.*, **168**(5):1165-8116 (1993), Piatak *et al.*, *Science*, **259**(5102):1749-1754 (1993), Gupta *et al.*, Ninth International Conference on AIDS/Fourth STD World Congress, June 7-11, 1993, Berlin, Germany, Saksela *et al.*, *Proc. Natl. Acad. Sci. USA*, **91**(3):1104-1108 (1994)). A method for accurately quantitating viral load would be useful.

In a multiplex assay, it is especially desirable that quantitative measurements of different targets accurately reflect the true ratio of the target sequences. However, the data obtained using multiplexed, exponential nucleic acid amplification methods is at best semi-quantitative. A number of factors are involved:

1. When a multiplex assay involves different priming events for different target sequences, the relative efficiency of these events may vary for different targets. This is due to the stability and structural differences between the various primers used.

2. If the rates of product strand renaturation differ for different targets, the extent of competition with priming events will not be the same for all targets.

3. For reactions involving multiple ligation events, such as LCR, there may be small but significant differences in the relative efficiency of ligation events for each target sequence. Since the ligation events are repeated many times, this effect is magnified.

4. For reactions involving reverse transcription (3SR, NASBA) or klenow strand displacement (SDA), the extent of polymerization processivity may differ among different target sequences.

5. For assays involving different replicatable RNA probes, the replication efficiency of each probe is usually not the same, and hence the probes compete unequally in replication reactions catalyzed by Qβ replicase.

6. A relatively small difference in yield in one cycle of amplification results in a large difference in amplification yield after several cycles. For example, in a PCR reaction with 25 amplification cycles and a 10% difference in yield per cycle, that is, 2-fold versus 1.8-fold amplification per cycle, the yield would be 2.0^{25}=33,554,000 versus 1.8^{25}=2,408,800. The difference in overall yield after 25 cycles is 14-fold. After 30 cycles of amplification, the yield difference would be more than 20-fold.

A method for amplifying and detecting nucleic acid sequences based on the presence of a specific target sequence using rolling circle replication is described in PCT Application WO 97/19193 by Yale University. In this method, a single stranded circular DNA molecule is replicated in an isothermal, continuous reaction to produce a single linear DNA molecule with numerous tandem repeats of the

complement of the sequence of the circular DNA molecule. Replication is dependent on the presence of a primer specific for the circular DNA molecule. In the method of WO 97/19193, the primer is coupled to a binding moiety, such as an oligonucleotide probe or an antibody, that can bind to a specific molecule, such as a nucleic acid sequence or a protein. By making the presence of the primer dependent on the presence of the specific molecule (that is, the analyte), replication of the circular DNA molecule, which requires the primer, is made dependent on the presence of the analyte. In this way, detection of the tandem repeat DNA is made a surrogate for the presence of the analyte. This method is not optimal for separate detection of closely related sequences, such as single-base mutations or alleles, since hybridization discrimination between traditional probes differing in a single nucleotide is difficult to achieve.

Current technologies for quantitative profiling of mRNA/cDNA expression levels in biological samples involve the use of either cDNA arrays (Schena *et al.*, *Proc. Natl Acad. Sci. USA*, **91**:10614-10619 (1994)) or high density oligonucleotide arrays (Lockhart *et al.*, *Nature Biotechnology*, **14**:1675-1680 (1996)). In the case of the cDNA arrays by Schena *et al.*, the detection of a single molecular species in each element of the array requires the presence of at least 100,000 bound target molecules. In the case of the DNA chip arrays used by Lockhart *et al.*, the detection limit for hybridized RNA is of the order of 2000 molecules.

Current technologies for detection of mutations in DNA include cloning and genetic screens, DNA sequencing (with or without cloning), Single Strand Conformational Polymorphism analysis (SSCP), Multiple Allele-Specific Detection Assay (MASDA), oligonucleotide arrays (DNA chips, such as Affymetrix), and ASO-PCR, or PCR plus genetic bit analysis with sequencing primers. Methods of detecting nucleotide sequences by ligating together two probes which hybridize to adjacent sequences in the target nucleic acid molecule are described in U.S. Patent Nos. 4,883,750, 5,242,794, and 5,521,065, all to Whiteley *et al.* These methods do not involve replication or other amplification of the signal generated by ligation. Of all these methods, only cloning and genetic screens, or cloning followed by DNA sequencing are capable of detecting somatic mutations that may occur at a level of one DNA strand in 10,000 wild type strands.

Estimates of the accumulation of point mutations at the HPRT locus in normal tissues of 70-year old individuals is of the order of 2×10^{-7} per nucleotide (Simpson, *Adv. Cancer Res.* **71**:209-240 (1997)). In tumor cells, the frequency may be 10 to 1000 times higher, depending on growth conditions (Richards *et al.*, *Science* **277**:1523-1526 (1997)). Thus, measurements of somatic mutation frequency in very early stages of cancer implies measuring mutation rates of the order of 2×10^{-6} to 2×10^{-5}. Such infrequent events are difficult to measure using previous technologies.

It would be desirable to measure mutation rates using automated procedures. DNA sequencing of cloned material is an option, but the cost of sequencing a million bases per patient sample is prohibitive with current technology. High density DNA "chips" (Lockhart *et al.*, 1996) have been used for mutation analysis (Hacia *et al.*, *Nature Genetics* **14**:441-447 (1997)). While the DNA chip and cDNA array technologies are capable of detecting mutations in 250,000 DNA loci simultaneously, the detection of somatic mutant DNA strands that represent less than 2% of the total DNA strands at a given locus is not possible. This is because the signals are generated by the averaging of thousands of hybridization events, and the mutant signal gets lost by dilution and cross-hybridization noise.

Accordingly, there is a need for nucleic acid detection methods that are both sensitive and can more easily distinguish between closely related sequences.

It is therefore an object of the disclosed invention to provide a method of detecting nucleic acid sequences that can discriminate between closely related sequences.

It is another object of the disclosed invention to provide a method of determining the amount of specific target nucleic acid sequences present in a sample where the number of signals measured is proportional to the amount of a target sequence in a sample and where the ratio of signals measured for different target sequences substantially matches the ratio of the amount of the different target sequences present in the sample.

It is another object of the disclosed invention to provide a method of detecting and determining the amount of multiple specific target nucleic acid sequences in a single sample where the ratio of signals measured for different

target nucleic acid sequences substantially matches the ratio of the amount of the different target nucleic acid sequences present in the sample.

It is another object of the disclosed invention to provide a method of detecting the presence of single copies of target nucleic acid sequences.

It is another object of the disclosed invention to provide a method of detecting the presence of target nucleic acid sequences representing individual alleles of a target genetic element.

It is another object of the disclosed invention to provide a method for detecting, and determining the relative amounts of, multiple molecules of interest in a sample.

SUMMARY OF THE INVENTION

Disclosed are compositions and a method for detecting single nucleic acid molecules using rolling circle amplification (RCA) of pre-formed single-stranded circular templates, referred to as amplification target circles. The disclosed method is highly sensitive, allowing detection of single molecules. This is accomplished through RCA which allows production of numerous copies of circular templates in a single, isothermic reaction. A single round of amplification using rolling circle replication results in a large amplification of the circularized probe sequences, orders of magnitude greater than a single cycle of PCR replication and other amplification techniques in which each cycle is limited to a doubling of the number of copies of a target sequence.

In one form of the method, referred to as bipartite primer rolling circle amplification (BP-RCA), RCA of the amplification target circle (ATC) depends on the formation of a primer by target-mediated ligation. In the presence of a nucleic acid molecule having the target sequence, a probe and a combination probe/primer oligonucleotide can hybridize to adjacent sites on the target sequence allowing the probes to be ligated together. By attaching the first probe to a substrate such as a bead or glass slide, unligated probe/primer can be removed after ligation. The only primers remaining will be primers ligated, via the probe portion of the probe/primer, to the first probe. The ligated primer can then be used to prime replication of its cognate ATC. In this way, an ATC will only be replicated if the target sequence (to which its cognate probe/primer is complementary) is present.

BP-RCA is useful, for example, for determining which target sequences are present in a nucleic acid sample, or for determining which samples contain a target sequence. The former can be accomplished, for example, by using a variety of probe sequences, each complementary to a different target sequence of interest, and different ATCs designed to be primed by only one of the primer sequences (present in a probe/primer). Probe/primers complementary to a given target sequence of interest will only be ligated to the immobilized first probe when that target sequence is present in the nucleic acid sample, and only those ATCs complementary to ligated primers will be amplified. As a result, only those ATCs corresponding to target sequences in the nucleic acid sample will be amplified, thus identifying the target sequences present.

Determining which samples contain a target sequence can be accomplished, for example, by using multiple copies of a single form of probe complementary to the target sequence of interest and multiple copies of a single form of ATC designed to be primed by the primer sequence (present in the probe/primer). Parallel assays can then be performed, each using the same probe, probe/primer, and ATC, where each assay uses a different sample. This can be accomplished, for example, by coating a glass slide with the probe, spotting the probe/primer in an array of spots on the slide, spotting a different sample on each of the array spots, and ligating. If a sample contains the target sequence of interest, the probe/primer will be ligated to the immobilized first probe in that assay spot and the ATC, added after washing away unligated probe/primer, will be amplified in assay spots where the target sequence was present.

In another form of the method, referred to as immobilized primer rolling circle amplification (IP-RCA), RCA of the ATC depends on incorporation of a target sequence in the ATC during its formation. If the target sequence has been incorporated, a primer that can hybridize to the sequence will prime RCA of the ATC. This form of the method is useful for determining which form or forms of a variable sequence are present in a nucleic acid sample. For example, if a particular gene can have one of three sequences in a particular location (such a one wild type sequence and two different mutant sequences), IP-RCA can be used to incorporate the critical region into an ATC followed by RCA of the ATC using one of three

primers, each specific for one of the possible sequences. The ATC will only be replicated if it contains the target sequence complementary to the specific primer.

IP-RCA is useful, for example, for determining which target sequences are present in a nucleic acid sample. This can be accomplished, for example, by using a variety of primer sequences, each complementary to a different target sequence of interest, and different open circle probes (OCPs) designed to form ATCs that can be primed by only one of the primer sequences. Priming will occur only if the proper target sequence is incorporated into the ATC during formation. A mixture of the OCPs designed for the various target sequences can first be mixed with a nucleic acid sample and then subjected to target-mediated, gap-filling ligation. This results in the formation of ATCs with sequences related to target sequences of interest that are present in the nucleic acid sample. The ATCs can then be spread over a slide containing an array of immobilized primers, each primer specific for a different target sequence, and an amplification reaction can be performed. ATCs will be amplified only at spots containing the primer corresponding to that ATC. Thus, the location of amplified products identifies which target sequences are present in the sample.

Following amplification of an ATC in the disclosed method, the amplified sequences can be detected and quantified using any of the conventional detection systems for nucleic acids such as detection of fluorescent labels, enzyme-linked detection systems, antibody-mediated label detection, and detection of radioactive labels. Major advantages of this method are that the ligation operation can be manipulated to obtain allelic discrimination, the amplification operation is isothermal, and signals are strictly quantitative because the amplification reaction is linear and is catalyzed by a highly processive enzyme.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of tandem sequence DNA (TS-DNA) and an address probe designed to hybridize to the portion of the TS-DNA corresponding to part of the right and left target probes of the open circle probe and the gap oligonucleotide. The TS-DNA is SEQ ID NO:2 and the address probe is SEQ ID NO:3.

Figure 2 is a diagram of an example of bipartite primer RCA (BP-RCA). A first primer (P1) is immobilized on a solid support (S) using a linker (L). A target sequence in a target molecule (T) complementary to the first probe is then

hybridized to the first probe. A second probe (P2), which is complementary to an adjacent region of the target sequence and which is coupled 5' end to 5' end with a primer (Pr), is then hybridized to the target sequence. The two probes (P1 and P2), which are hybridized adjacent to each other on the target sequence are then ligated together. The target (T) is then washed from the probes and a circular template (CT), also referred to as an amplification target circle (ATC), is hybridized to the now immobilized primer (Pr). The circular template is then subjected to rolling circle amplification primed by the primer. The resulting amplified product, which remains attached to the solid support via the primer, the two probes and the linker, is then subjected to collapse and detection.

Figure 3 is a diagram of an example of a probe/primer and half probe hybridized to a target sequence. The diagram shows the relationship between the target sequence and the right and left target probes.

Figure 4 is a diagram of an example of a gap oligonucleotide, probe/primer and half probe hybridized to a target sequence. The diagram shows the relationship between the target sequence, the gap oligonucleotide, and the right and left target probes.

DETAILED DESCRIPTION OF THE INVENTION

Disclosed are compositions and a method for detecting single nucleic acid molecules using rolling circle amplification (RCA) of pre-formed single-stranded circular templates, referred to as amplification target circles. The disclosed method is highly sensitive, allowing detection of single molecules. This is accomplished through RCA which allows production of numerous copies of circular templates in a single, isothermic reaction. A single round of amplification using rolling circle replication results in a large amplification of the circularized probe sequences, orders of magnitude greater than a single cycle of PCR replication and other amplification techniques in which each cycle is limited to a doubling of the number of copies of a target sequence.

In one form of the method, referred to as bipartite primer rolling circle amplification (BP-RCA), RCA of the amplification target circle (ATC) depends on the formation of a primer by target-mediated ligation. In the presence of a nucleic acid molecule having the target sequence, a probe and a combination probe/primer oligonucleotide can hybridize to adjacent sites on the target sequence allowing the

probes to be ligated together. By attaching the first probe to a substrate such as a bead or glass slide, unligated probe/primer can be removed after ligation. The only primers remaining will be primers ligated, via the probe portion of the probe/primer, to the first probe. The ligated primer can then be used to prime replication of its cognate ATC. In this way, an ATC will only be replicated if the target sequence (to which its cognate probe/primer is complementary) is present.

BP-RCA is useful, for example, for determining which target sequences are present in a nucleic acid sample, or for determining which samples contain a target sequence. The former can be accomplished, for example, by using a variety of probe sequences, each complementary to a different target sequence of interest, and different ATCs designed to be primed by only one of the primer sequences (present in a probe/primer). Probe/primers complementary to a given target sequence of interest will only be ligated to the immobilized first probe when that target sequence is present in the nucleic acid sample, and only those ATCs complementary to ligated primers will be amplified. As a result, only those ATCs corresponding to target sequences in the nucleic acid sample will be amplified, thus identifying the target sequences present.

Determining which samples contain a target sequence can be accomplished, for example, by using multiple copies of a single form of probe complementary to the target sequence of interest and multiple copies of a single form of ATC designed to be primed by the primer sequence (present in the probe/primer). Parallel assays can then be performed, each using the same probe, probe/primer, and ATC, where each assay uses a different sample. This can be accomplished, for example, by coating a glass slide with the probe, spotting the probe/primer in an array of spots on the slide, spotting a different sample on each of the array spots, and ligating. If a sample contains the target sequence of interest, the probe/primer will be ligated to the immobilized first probe in that assay spot and the ATC, added after washing away unligated probe/primer, will be amplified in assay spots where the target sequence was present.

The amplification of small circularized oligonucleotides by BP-RCA is rapid, technically simple, and the amplified DNA will not diffuse away from the site of synthesis. RCA products may be detected by incorporating happens or fluors directly, and it is possible to use circles with biased base compositions to

obtain differential labeling. A larger range of labeling combinations is attainable by collapsing the amplified DNA. For DNA microarray applications, one may use any two-color system which permits measurements of relative allele frequencies by single molecule counting. The two-circle/two-primer signal generating system shown in Figure 2 and described in Example 3 is extensible to any number of arrayed probes, while retaining the use of the same pair of amplification target circles. Two different allele-discriminating primers should be used for each mutational locus being assayed.

While photolithographic DNA microarray technology enables massively parallel assays for mutation detection, altered bases are only detectable using prior methods of detection if they constitute a sizable fraction of the DNA population. Thus, such arrays are well suited for the detection of germline mutations, but not rare somatic mutations when using prior methods of detection. BP-RCA extends the utility of DNA microarrays by permitting the detection of infrequent mutations in the presence of an excess of wild-type DNA. The detection limit for such mutations will be determined by the stringency of the DNA ligation reaction, which should be improved by using new ligase variants (Luo *et al.*, *Nucl. Acids Res.* **24**:3071-3078 (1996)).

BP-RCA is also useful for mRNA profiling, where standard cDNA hybridization approaches may not be sensitive enough to detect changes in the concentration of low abundance gene products. The single molecule counting approach is both sensitive and linear in its response to target concentration. BP-RCA can also be used with a multiparametric color coding such as Combinatorial Multicolor Coding (Speicher *et al.*, *Nature Genet.* **12**:368-375 (1996)). For example, a 6-fluor tagging approach would permit the simultaneous identification of 63 different types of signals on a surface by virtue of their unique spectral signatures, enabling powerful multiplex BP-RCA assays.

In another form of the method, referred to as immobilized primer rolling circle amplification (IP-RCA), RCA of the ATC depends on incorporation of a target sequence in the ATC during its formation. If the target sequence has been incorporated, a primer that can hybridize to the sequence will prime RCA of the ATC. This form of the method is useful for determining which form or forms of a variable sequence are present in a nucleic acid sample. For example, if a particular

gene can have one of three sequences in a particular location (such a one wild type sequence and two different mutant sequences), IP-RCA can be used to incorporate the critical region into an ATC followed by RCA of the ATC using one of three primers, each specific for one of the possible sequences. The ATC will only be replicated if it contains the target sequence complementary to the specific primer.

IP-RCA is useful, for example, for determining which target sequences are present in a nucleic acid sample. This can be accomplished, for example, by using a variety of primer sequences, each complementary to a different target sequence of interest, and different open circle probes (OCPs) designed to form ATCs that can be primed by only one of the primer sequences. Priming will occur only if the proper target sequence is incorporated into the ATC during formation. A mixture of the OCPs designed for the various target sequences can first be mixed with a nucleic acid sample and then subjected to target-mediated, gap-filling ligation. This results in the formation of ATCs with sequences related to target sequences of interest that are present in the nucleic acid sample. The ATCs can then be spread over a slide containing an array of immobilized primers, each primer specific for a different target sequence, and an amplification reaction can be performed. ATCs will be amplified only at spots containing the primer corresponding to that ATC. Thus, the location of amplified products identifies which target sequences are present in the sample.

Another form of the disclosed method makes use of a 3'-3' probe/primer to detect RNA or DNA targets *in situ*. A 3'-3' probe/primer may be used to detect RNA or DNA molecules on a surface by means of a two-step process that does not involve a ligation step. Effective anchoring of the probe to the target is achieved by means of primer extension reaction. An example of this method of detection is outlined below.

1. A probe/primer containing two 3' ends (target probe portion and primer portion coupled head to head) is prepared by a synthesis in which the first 28 bases are standard phosphoramidites, followed by a spacer of six standard phosphoramidite T residues, and then the next 20 bases added comprise reversed phosphoramidites (dA-5'-CE Phosphoramidite, dT-5'-CE Phosphoramidite, dC-5'-CE Phosphoramidite, dG-5'-CE Phosphoramidite; Glen Research, Sterling, Virginia). The first 28 bases on one 3'-end of the probe/primer are designed to

function as a rolling circle replication primer (this is the primer portion), while the last 20 bases on the other 3'-end of the probe/primer are designed to function as a target probe portion which will prime extension synthesis.

2. The probe/primer is hybridized and extended on the surface of a glass slide containing target molecules. The target molecules may be RNA or DNA, and the targets may be present in cells (cytological preparations) or may be bound on the glass surface as naked (deproteinized) RNA or DNA. Primer extension is performed using any DNA polymerase capable of supporting primer extension in the presence of dNTPs. Thermostable enzymes are preferred. For DNA targets, the enzyme may be, for example, Taq DNA polymerase, Vent DNA polymerase, Pfu DNA polymerase, or Thermus flavus DNA polymerase. For RNA targets, a thermostable reverse transcriptase capable of supporting primer extension is preferred. Suitable enzymes are Thermus thermophilus DNA polymerase (in the presence of manganese, which permits the enzyme to copy RNA) and avian Thermo-Script reverse transcriptase (Life Technologies, Inc, Rockville, Maryland). During this reaction, the 20-base probe portion of the probe/primer binds to its complementary sequence and is extended anywhere from 50 to several thousand nucleotides. The extension reaction causes the probe/primer to bind very tightly to its target.

3. The glass slide is washed to remove the excess unbound (and unextended) probe/primers, and amplification target circles complementary to the 28-base rolling circle replication primer portion are added. In the presence of a strand-displacing DNA polymerase, such as Sequenase 2.0, ø29 DNA polymerase, Bst large fragment DNA polymerase, or Vent exo(-) DNA polymerase, the rolling circle replication primer is extended to generated a long tandem sequence DNA, which remains bound to the target site of the original primer extension event of step 2.

4. The tandem sequence DNA can then be labeled and collapsed into a single point source, using any of the labeling and collapsing methods described herein. This allows detection of target nucleic acids in situ, that is, at the location where the target nucleic acid is located.

Use of a primer extension reaction can also be used to discriminate between two or more forms of a sequence that differ at particular nucleotide position(s). For

example, different alleles of a gene differ at some nucleotide positions. By using probe/primers where the 3' terminal nucleotide of the target probe portion is designed to overlap a variable nucleotide position, primer extension will be dependent on hybridization between the target sequence at this position and the 3' terminal nucleotide in the probe sequence. That is, if the 3' terminal nucleotide in the probe sequence is not complementary to the nucleotide opposite it when the probe/primer hybridizes to the target sequence, primer extension will be hampered. A particular form of a sequence (a particular allele of a gene, for example) can be detected using the disclosed method by using a probe/primer where the target probe sequence ends in a nucleotide complementary to the nucleotide present in the sequence of interest (with the rest of the probe sequence complementary to the adjacent nucleotides in the target sequence). The probe will be extended only when hybridized to a target sequence having the desired nucleotide at the critical position.

Following amplification of an ATC in the disclosed method, the amplified sequences can be detected and quantified using any of the conventional detection systems for nucleic acids such as detection of fluorescent labels, enzyme-linked detection systems, antibody-mediated label detection, and detection of radioactive labels. Major advantages of this method are that the ligation operation can be manipulated to obtain allelic discrimination, the amplification operation is isothermal, and signals are strictly quantitative because the amplification reaction is linear and is catalyzed by a highly processive enzyme.

I. Materials

A. Rolling Circle Replication Primer

A rolling circle replication primer (RCRP) is an oligonucleotide having sequence complementary to the primer complement portion of an open circle probe (OCP) or amplification target circle (ATC). This sequence is referred to as the complementary portion of the RCRP. The complementary portion of a RCRP and the cognate primer complement portion can have any desired sequence so long as they are complementary to each other. In general, the sequence of the RCRP can be chosen such that it is not significantly complementary to any other portion of the OCP or ATC. The complementary portion of a rolling circle replication primer can be any length that supports specific and stable hybridization between the primer

and the primer complement portion. Generally this is 10 to 35 nucleotides long, but is preferably 16 to 20 nucleotides long.

It is preferred that rolling circle replication primers also contain additional sequence at the 5' end of the RCRP that is not complementary to any part of the OCP or ATC. This sequence is referred to as the non-complementary portion of the RCRP. The non-complementary portion of the RCRP, if present, serves to facilitate strand displacement during DNA replication. The non-complementary portion of a RCRP may be any length, but is generally 1 to 100 nucleotides long, and preferably 4 to 8 nucleotides long. A preferred form of non-complementary portion is the probe portion of a probe/primer. The rolling circle replication primer may also include modified nucleotides to make it resistant to exonuclease digestion. For example, the primer can have three or four phosphorothioate linkages between nucleotides at the 5' end of the primer. Such nuclease resistant primers allow more stable primers for immobilization on solid substrates. Such nuclease resistant primers also allow selective degradation of excess unligated OCP and gap oligonucleotides that might otherwise interfere with hybridization of detection probes, address probes, and secondary OCPs to the amplified nucleic acid. A rolling circle replication primer can be used as the tertiary DNA strand displacement primer in strand displacement cascade amplification.

Rolling circle replication primers can be immobilized on a solid-state support or substrate. This allows the tandem sequence DNA formed by amplification primed by the rolling circle replication primer to be immobilized via the rolling circle replication primer. Immobilized rolling circle replication primers are used in immobilized primer rolling circle amplification (IP-RCA).

1. Bipartite primers

Bipartite primers are rolling circle replication primers formed by ligation of a pair of oligonucleotide molecules. The pair of oligonucleotide molecules is made up of a probe/primer and a half probe. As used herein, a probe/primer is an oligonucleotide containing a primer portion and a target probe portion. The primer portion of a probe/primer corresponds to the complementary portion of a rolling circle replication primer as described above. That is, for example, the primer portion of a probe/primer is complementary to the primer complement portion of

an OCP or ATC and can serve to prime rolling circle replication of an ATC. Half probes contain a target probe portion.

The target probe portion of a probe/primer and the target probe portion of the half probe are complementary to different portions of a target sequence. The target probe portions can each be any length that supports specific and stable hybridization between the target probes and the target sequence. For this purpose, a length of 10 to 35 nucleotides for each target probe portion is preferred, with target probe portions 15 to 20 nucleotides long being most preferred. The target probe portion of the probe/primer is referred to as the left target probe, and the target probe portion of the half probe is referred to as the right target probe. These target probe portions are also referred to herein as left and right target probes or left and right probes. The target probe portions are complementary to a target nucleic acid sequence.

The target probe portions of the probe/primer and the half probe are complementary to the target sequence, such that upon hybridization an end of the right target probe portion and an end of the left target probe portion are base-paired to adjacent nucleotides in the target sequence, such that they serve as a substrate for ligation (Figure 3). This requires that the target probe portions be hybridized with 3' and 5' ends adjacent to each other. Preferably, the 5 end of the right target probe portion and the 3 end of the left target probe portion are base-paired to adjacent nucleotides in the target sequence (Figure 3).

Optionally, the target probe portions may hybridize in such a way that they are separated by a gap space. In this case the target probe portion of the probe/primer and the target probe portion of the half probe may only be ligated if one or more additional oligonucleotides, referred to as gap oligonucleotides, are used, or if the gap space is filled during the ligation operation. The gap oligonucleotides hybridize to the target sequence in the gap space to form a continuous probe/target hybrid (Figure 4). The gap space may be any length desired but is generally ten nucleotides or less. It is preferred that the gap space is between about three to ten nucleotides in length, with a gap space of four to eight nucleotides in length being most preferred. Alternatively, a gap space could be filled using a DNA polymerase during the ligation operation. When using such a gap-filling operation, a gap space of three to five nucleotides in length is most

preferred. As another alternative, the gap space can be partially bridged by one or more gap oligonucleotides, with the remainder of the gap filled using DNA polymerase.

It is preferred that the probe/primer be constructed such that the primer portion and the target probe portion are in opposite orientations, joined 5' end to 5' end (head to head). This gives the probe/primer two 3' ends and allows the 3' end of the target probe portion of the probe/primer to be ligated to the 5' end of the half probe while leaving a free 3' end on the primer portion (a free 3' end is required for the primer portion to function as a primer). This arrangement is preferred because it can prevent unintentional priming of amplification target circle replication by the half probe when the half probe is immobilized on a solid support or coupled to another molecule via its 3' end.

Head to head probe/primers can be made by coupling the 5' ends of a probe oligonucleotide and a primer oligonucleotide using any suitable or known method of coupling. The primer and probe oligonucleotides can be coupled using a linker molecule. It is preferred that such a linker molecule be made up of nucleotides. It is preferred that head to head probe/primers be synthesized as a single oligonucleotide using first standard phosphoramidite nucleotides for the first portion and then switching to reversed phosphoramidite nucleotides, such as dN-5'-CE phosphoramidites (Glen Research, Sterling, VA), for the second portion. This allows synthesis using standard chemistry in a continuous synthesis operation.

One form of probe/primers, referred to as randomized probe/primers, makes use of a target probe portion with a partially random sequence and universal nucleosides. Such a probe/primer is useful for detecting target sequences in a sample with many potential targets. The partially random sequence is at the 3' end of the target probe portion and includes one specific nucleotide (A, C, G, or T) at 3' end next to randomized nucleotides. The number of randomized nucleotides can be any number that allows the ligase to use the 3' end nucleotide as a ligation substrate. Preferably the number of randomized nucleotides is limited to reduce the sequence complexity of the probe/primer. It is preferred that three, four, five, or six randomized nucleotides be used. It is more preferred that four randomized nucleotides be used.

The universal nucleosides, such as nitropyrrole (Nichols *et al.*, *Nature* 369:492-493 (1994)) or nitroindole (Loakes and Brown, *Nucleic Acids Res.* 22:4039-4043 (1994)), can interact with any nucleotide and increase the efficiency of ligation by providing increased hybrid stability without any sequence requirement. The universal nucleotides are included 5' of the randomized nucleotides. An example of four varieties of randomized target probes is as follows, where n is a universal nucleotide and N is a mixture of the four nucleotides, A, C, G, and T.

nnnnNNNNA-3'

nnnnNNNNC-3'

nnnnNNNNG-3'

nnnnNNNNT-3'

The presence of a specific nucleotide at the 3' end of the target probe portion results in four different varieties of randomized probe/primers, each with a different 3' end nucleotide. Hybridization of this 3' end nucleotide to the target sequence is required for ligation to occur. This makes each of the four varieties of randomized probe/primers capable of being ligated only in the presence of a target sequence having the appropriate complementary nucleotide. Thus, each of the four varieties of randomized probe/primers is ligatable in the presence of different target sequences. The effect is that a universal set of randomized probe/primers (a set of each of the four varieties of randomized probe/primers), in combination with half probes having appropriate sequences complementary to target sequences of interest, can be used to mediate generation of an amplified signal from any target sequence.

A preferred form of probe/primer has the structure

3'-Pr-L-n_jN_kX-3'

where Pr is the primer portion, n_jN_kX is the target probe portion, L is either a covalent bond or a linker between the primer portion and the target probe portion, n represents a universal nucleoside, j is 2-20, N represents one of the nucleotides A, C, G or T, k is 3, 4, 5, 6, 7, or 8, and X represents one of the nucleotides A, C, G or T. Probe/primers of this structure can be used in sets of primers where each N is randomized within each set of probe/primers, and where X represents the same nucleotide in all of the probe/primers in a set of

probe/primers. By using fours different sets of this type, where X represents a different nucleotide in each of the four sets, such primers can be used as universal probe/primers to detect any target sequence (using a half probe of appropriate sequence).

The primer portion of each of the four varieties of randomized probe/primers can be a different sequence to allow separate detection of the amplification product of each of the randomized probe/primer varieties. This is preferably accomplished by using four different ATCs, each primed by only one of the four varieties of randomized probe/primers. The ATC amplified will be indicative of which specific nucleotide was at the 3' end of the probe/primer that became ligated. The universal set of randomized probe/primers, and the matching amplification target circles, can be used with any set of half probes. Thus, a reagent kit including a set of the four varieties of randomized probe/primes and a set of the four matching ATCs can be used to detect target sequences hybridized by any half probe or combination of half probes. Immobilized half probe sets and arrays with any desired combination of half probes targeted to any desired combination of target sequences can be used with such a universal reagent kit to detect any target sequence.

Another benefit of randomized probe/primer sets is that any number and complexity of target sequences can be probed using BP-RCA without the negative consequences of an increasingly low concentration of probe as the number of targets increases. That is, if specific primer/probes having specific target probe portion sequences were used for each target sequence to be probed, it becomes difficult to get a sufficient concentration of each probe in solution to allow hybridization to occur with reasonable efficiency when the number of target sequences becomes large. Using randomized probe/primers having a complexity of, for example, 256 (the complexity when four randomized nucleotides are used), a concentration of one to five micromolar for each of the four varieties of randomized probe/primers give a sufficient solution concentration of each of the probe sequences.

B. Amplification Target Circles

An amplification target circle (ATC) is a circular single-stranded DNA molecule, generally containing between 40 to 1000 nucleotides, preferably between

about 50 to 150 nucleotides, and most preferably between about 50 to 100 nucleotides. Portions of ATCs have specific functions making the ATC useful for rolling circle amplification (RCA). These portions are referred to as the primer complement portion, the detection tag portions, the secondary target sequence portions, the address tag portions, and the promoter portion. The primer complement portion is a required element of an amplification target circle. Detection tag portions, secondary target sequence portions, address tag portions, and promoter portions are optional. Generally, an amplification target circle is a single-stranded, circular DNA molecule comprising a primer complement portion. Those segments of the ATC that do not correspond to a specific portion of the ATC can be arbitrarily chosen sequences. It is preferred that ATCs do not have any sequences that are self-complementary. It is considered that this condition is met if there are no complementary regions greater than six nucleotides long without a mismatch or gap. It is also preferred that ATCs containing a promoter portion do not have any sequences that resemble a transcription terminator, such as a run of eight or more thymidine nucleotides. Ligated open circle probes are a type of ATC, and as used herein the term amplification target circle includes ligated open circle probes. An ATC can be used in the same manner as described herein for OCPs that have been ligated.

An amplification target circle, when replicated, gives rise to a long DNA molecule containing multiple repeats of sequences complementary to the amplification target circle. This long DNA molecule is referred to herein as tandem sequences DNA (TS-DNA). TS-DNA contains sequences complementary to the primer complement portion and, if present on the amplification target circle, the detection tag portions, the secondary target sequence portions, the address tag portions, and the promoter portion. These sequences in the TS-DNA are referred to as primer sequences (which match the sequence of the rolling circle replication primer), spacer sequences (complementary to the spacer region), detection tags, secondary target sequences, address tags, and promoter sequences. Amplification target circles are useful as tags for specific binding molecules.

1. Primer Complement Portion

The primer complement portion of an amplification target circle is complementary to the rolling circle replication primer (RCRP). Each ATC should

have a single primer complement portion. This allows rolling circle replication to initiate at a single site on ATCs. The primer complement portion and the cognate primer can have any desired sequence so long as they are complementary to each other. In general, the sequence of the primer complement can be chosen such that it is not significantly similar to any other portion of the ATC. The primer complement portion can be any length that supports specific and stable hybridization between the primer complement portion and the primer. For this purpose, a length of 10 to 35 nucleotides is preferred, with a primer complement portion 16 to 20 nucleotides long being most preferred.

2. Detection Tag Portions

Detection tag portions of an amplification target circle have sequences matching the sequence of the complementary portion of detection probes. These detection tag portions, when amplified during rolling circle replication, result in TS-DNA having detection tag sequences that are complementary to the complementary portion of detection probes. If present, there may be one, two, three, or more than three detection tag portions on an ATC. It is preferred that an ATC have two, three or four detection tag portions. Most preferably, an ATC will have three detection tag portions. Generally, it is preferred that an ATC have 60 detection tag portions or less. There is no fundamental limit to the number of detection tag portions that can be present on an ATC except the size of the ATC. When there are multiple detection tag portions, they may have the same sequence or they may have different sequences, with each different sequence complementary to a different detection probe. It is preferred that an ATC contain detection tag portions that have the same sequence such that they are all complementary to a single detection probe. For some multiplex detection methods, it is preferable that ATCs contain up to six detection tag portions and that the detection tag portions have different sequences such that each of the detection tag portions is complementary to a different detection probe. The detection tag portions can each be any length that supports specific and stable hybridization between the detection tags and the detection probe. For this purpose, a length of 10 to 35 nucleotides is preferred, with a detection tag portion 15 to 20 nucleotides long being most preferred.

3. Secondary Target Sequence Portions

Secondary target sequence portions of an amplification target circle have sequences matching the sequence of target probes of a secondary open circle probe. These secondary target sequence portions, when amplified during rolling circle replication, result in TS-DNA having secondary target sequences that are complementary to target probes of a secondary open circle probe. If present, there may be one, two, or more than two secondary target sequence portions on an ATC. It is preferred that an ATC have one or two secondary target sequence portions. Most preferably, an ATC will have one secondary target sequence portion. Generally, it is preferred that an ATC have 50 secondary target sequence portions or less. There is no fundamental limit to the number of secondary target sequence portions that can be present on an ATC except the size of the ATC. When there are multiple secondary target sequence portions, they may have the same sequence or they may have different sequences, with each different sequence complementary to a different secondary OCP. It is preferred that an ATC contain secondary target sequence portions that have the same sequence such that they are all complementary to a single target probe portion of a secondary OCP. The secondary target sequence portions can each be any length that supports specific and stable hybridization between the secondary target sequence and the target sequence probes of its cognate OCP. For this purpose, a length of 20 to 70 nucleotides is preferred, with a secondary target sequence portion 30 to 40 nucleotides long being most preferred. As used herein, a secondary open circle probe is an open circle probe where the target probe portions match or are complementary to secondary target sequences in another open circle probe or an amplification target circle. It is contemplated that a secondary open circle probe can itself contain secondary target sequences that match or are complementary to the target probe portions of another secondary open circle probe. Secondary open circle probes related to each other in this manner are referred to herein as nested open circle probes.

4. Address Tag Portion

The address tag portion of an amplification target circle has a sequence matching the sequence of the complementary portion of an address probe. This address tag portion, when amplified during rolling circle replication, results in TS-

DNA having address tag sequences that are complementary to the complementary portion of address probes. If present, there may be one, or more than one, address tag portions on an ATC. It is preferred that an ATC have one or two address tag portions. Most preferably, an ATC will have one address tag portion. Generally, it is preferred that an ATC have 50 address tag portions or less. There is no fundamental limit to the number of address tag portions that can be present on an ATC except the size of the ATC. When there are multiple address tag portions, they may have the same sequence or they may have different sequences, with each different sequence complementary to a different address probe. It is preferred that an ATC contain address tag portions that have the same sequence such that they are all complementary to a single address probe. Preferably, the address tag portion overlaps all or a portion of the target probe portions, and all of any intervening gap space (Figure 1). Most preferably, the address tag portion overlaps all or a portion of both the left and right target probe portions. The address tag portion can be any length that supports specific and stable hybridization between the address tag and the address probe. For this purpose, a length between 10 and 35 nucleotides long is preferred, with an address tag portion 15 to 20 nucleotides long being most preferred.

5. Promoter Portion

The promoter portion corresponds to the sequence of an RNA polymerase promoter. A promoter portion can be included in an amplification target circle so that transcripts can be generated from TS-DNA. The sequence of any promoter may be used, but simple promoters for RNA polymerases without complex requirements are preferred. It is also preferred that the promoter is not recognized by any RNA polymerase that may be present in the sample containing the target nucleic acid sequence. Preferably, the promoter portion corresponds to the sequence of a T7 or SP6 RNA polymerase promoter. The T7 and SP6 RNA polymerases are highly specific for particular promoter sequences. Other promoter sequences specific for RNA polymerases with this characteristic would also be preferred. Because promoter sequences are generally recognized by specific RNA polymerases, the cognate polymerase for the promoter portion of the ATC should be used for transcriptional amplification. Numerous promoter sequences are known and any promoter specific for a suitable RNA polymerase can be used. The

promoter portion can be located anywhere on an ATC and can be in either orientation. Preferably, the promoter portion is oriented to promote transcription in the 5' to 3' direction of the amplification target circle. This orientation results in transcripts that are complementary to TS-DNA, allowing independent detection of TS-DNA and the transcripts, and prevents transcription from interfering with rolling circle replication.

C. Open Circle Probes

An open circle probe (OCP) is a linear single-stranded DNA molecule, generally containing between 50 to 1000 nucleotides, preferably between about 60 to 150 nucleotides, and most preferably between about 70 to 100 nucleotides. The OCP has a 5' phosphate group and a 3' hydroxyl group. This allows the ends to be ligated using a DNA ligase, or extended in a gap-filling operation. Portions of the OCP have specific functions making the OCP useful for RCA and LM-RCA. These portions are referred to as the target probe portions, the primer complement portion, the spacer region, the detection tag portions, the secondary target sequence portions, the address tag portions, and the promoter portion. The target probe portions and the primer complement portion are required elements of an open circle probe. The primer complement portion is part of the spacer region. Detection tag portions, secondary target sequence portions, and promoter portions are optional and, when present, are part of the spacer region. Address tag portions are optional and, when present, may be part of the spacer region. Generally, an open circle probe is a single-stranded, linear DNA molecule comprising, from 5' end to 3' end, a 5' phosphate group, a right target probe portion, a spacer region, a left target probe portion, and a 3' hydroxyl group, with a primer complement portion present as part of the spacer region. Those segments of the spacer region that do not correspond to a specific portion of the OCP can be arbitrarily chosen sequences. It is preferred that OCPs do not have any sequences that are self-complementary. It is considered that this condition is met if there are no complementary regions greater than six nucleotides long without a mismatch or gap. It is also preferred that OCPs containing a promoter portion do not have any sequences that resemble a transcription terminator, such as a run of eight or more thymidine nucleotides.

The open circle probe, when ligated and replicated, gives rise to a long DNA molecule containing multiple repeats of sequences complementary to the open circle probe. This long DNA molecule is referred to herein as tandem sequences DNA (TS-DNA). TS-DNA contains sequences complementary to the target probe portions, the primer complement portion, the spacer region, and, if present on the open circle probe, the detection tag portions, the secondary target sequence portions, the address tag portions, and the promoter portion. These sequences in the TS-DNA are referred to as target sequences (which match the original target sequence), primer sequences (which match the sequence of the rolling circle replication primer), spacer sequences (complementary to the spacer region), detection tags, secondary target sequences, address tags, and promoter sequences.

A particularly preferred embodiment is an open circle probe of 70 to 100 nucleotides including a left target probe of 20 nucleotides and a right target probe of 20 nucleotides. The left target probe and right target probe hybridize to a target sequence leaving a gap of five nucleotides, which is filled by a single pentanucleotide gap oligonucleotide.

1. Target Probe Portions

There are two target probe portions on each OCP, one at each end of the OCP. The target probe portions can each be any length that supports specific and stable hybridization between the target probes and the target sequence. For this purpose, a length of 10 to 35 nucleotides for each target probe portion is preferred, with target probe portions 15 to 20 nucleotides long being most preferred. The target probe portion at the 3' end of the OCP is referred to as the left target probe, and the target probe portion at the 5' end of the OCP is referred to as the right target probe. These target probe portions are also referred to herein as left and right target probes or left and right probes. The target probe portions are complementary to a target nucleic acid sequence.

The target probe portions are complementary to the target sequence, such that upon hybridization the 5' end of the right target probe portion and the 3' end of the left target probe portion are base-paired to adjacent nucleotides in the target sequence, with the objective that they serve as a substrate for ligation. Optionally, the 5' end and the 3' end of the target probe portions may hybridize in such a way

that they are separated by a gap space. In this case the 5' end and the 3' end of the OCP may only be ligated if one or more additional oligonucleotides, referred to as gap oligonucleotides, are used, or if the gap space is filled during the ligation operation. The gap oligonucleotides hybridize to the target sequence in the gap space to a form continuous probe/target hybrid. The gap space may be any length desired but is generally ten nucleotides or less. It is preferred that the gap space is between about three to ten nucleotides in length, with a gap space of four to eight nucleotides in length being most preferred. Alternatively, a gap space could be filled using a DNA polymerase during the ligation operation. When using such a gap-filling operation, a gap space of three to five nucleotides in length is most preferred. As another alternative, the gap space can be partially bridged by one or more gap oligonucleotides, with the remainder of the gap filled using DNA polymerase.

2. Primer Complement Portion

The primer complement portion of an open circle probe is complementary to the rolling circle replication primer (RCRP). Each OCP should have a single primer complement portion. This allows rolling circle replication to initiate at a single site on ligated OCPs. The primer complement portion and the cognate primer can have any desired sequence so long as they are complementary to each other. In general, the sequence of the primer complement can be chosen such that it is not significantly similar to any other portion of the OCP. The primer complement portion can be any length that supports specific and stable hybridization between the primer complement portion and the primer. For this purpose, a length of 10 to 35 nucleotides is preferred, with a primer complement portion 16 to 20 nucleotides long being most preferred. The primer complement portion can be located anywhere within the OCP. For use in IP-RCA, it is preferred that the primer complement portion is complementary to a sequence in a gap space in the OCP. This allows primer-specific amplification of an OCP that has incorporated into the gap space a specific sequence, such as the sequence of a specific target sequence.

The primer complement portion can also be part of the spacer region. In this case, it is preferred that the primer complement portion is adjacent to the right target probe, with the right target probe portion and the primer complement portion

preferably separated by three to ten nucleotides, and most preferably separated by six nucleotides. This location prevents the generation of any other spacer sequences, such as detection tags and secondary target sequences, from unligated open circle probes during DNA replication.

3. Detection Tag Portions

Detection tag portions are part of the spacer region of an open circle probe. Detection tag portions have sequences matching the sequence of the complementary portion of detection probes. These detection tag portions, when amplified during rolling circle replication, result in TS-DNA having detection tag sequences that are complementary to the complementary portion of detection probes. If present, there may be one, two, three, or more than three detection tag portions on an OCP. It is preferred that an OCP have two, three or four detection tag portions. Most preferably, an OCP will have three detection tag portions. Generally, it is preferred that an OCP have 60 detection tag portions or less. There is no fundamental limit to the number of detection tag portions that can be present on an OCP except the size of the OCP. When there are multiple detection tag portions, they may have the same sequence or they may have different sequences, with each different sequence complementary to a different detection probe. It is preferred that an OCP contain detection tag portions that have the same sequence such that they are all complementary to a single detection probe. For some multiplex detection methods, it is preferable that OCPs contain up to six detection tag portions and that the detection tag portions have different sequences such that each of the detection tag portions is complementary to a different detection probe. The detection tag portions can each be any length that supports specific and stable hybridization between the detection tags and the detection probe. For this purpose, a length of 10 to 35 nucleotides is preferred, with a detection tag portion 15 to 20 nucleotides long being most preferred.

4. Secondary Target Sequence Portions

Secondary target sequence portions are part of the spacer region of an open circle probe. Secondary target sequence portions have sequences matching the sequence of target probes of a secondary open circle probe. These secondary target sequence portions, when amplified during rolling circle replication, result in TS-DNA having secondary target sequences that are complementary to target probes

of a secondary open circle probe. If present, there may be one, two, or more than two secondary target sequence portions on an OCP. It is preferred that an OCP have one or two secondary target sequence portions. Most preferably, an OCP will have one secondary target sequence portion. Generally, it is preferred that an OCP have 50 secondary target sequence portions or less. There is no fundamental limit to the number of secondary target sequence portions that can be present on an OCP except the size of the OCP. When there are multiple secondary target sequence portions, they may have the same sequence or they may have different sequences, with each different sequence complementary to a different secondary OCP. It is preferred that an OCP contain secondary target sequence portions that have the same sequence such that they are all complementary to a single target probe portion of a secondary OCP. The secondary target sequence portions can each be any length that supports specific and stable hybridization between the secondary target sequence and the target sequence probes of its cognate OCP. For this purpose, a length of 20 to 70 nucleotides is preferred, with a secondary target sequence portion 30 to 40 nucleotides long being most preferred. As used herein, a secondary open circle probe is an open circle probe where the target probe portions match or are complementary to secondary target sequences in another open circle probe or an amplification target circle. It is contemplated that a secondary open circle probe can itself contain secondary target sequences that match or are complementary to the target probe portions of another secondary open circle probe. Secondary open circle probes related to each other in this manner are referred to herein as nested open circle probes.

5. Address Tag Portion

The address tag portion is part of either the target probe portions or the spacer region of an open circle probe. The address tag portion has a sequence matching the sequence of the complementary portion of an address probe. This address tag portion, when amplified during rolling circle replication, results in TS-DNA having address tag sequences that are complementary to the complementary portion of address probes. If present, there may be one, or more than one, address tag portions on an OCP. It is preferred that an OCP have one or two address tag portions. Most preferably, an OCP will have one address tag portion. Generally, it is preferred that an OCP have 50 address tag portions or less. There is no

fundamental limit to the number of address tag portions that can be present on an OCP except the size of the OCP. When there are multiple address tag portions, they may have the same sequence or they may have different sequences, with each different sequence complementary to a different address probe. It is preferred that an OCP contain address tag portions that have the same sequence such that they are all complementary to a single address probe. Preferably, the address tag portion overlaps all or a portion of the target probe portions, and all of any intervening gap space (Figure 1). Most preferably, the address tag portion overlaps all or a portion of both the left and right target probe portions. The address tag portion can be any length that supports specific and stable hybridization between the address tag and the address probe. For this purpose, a length between 10 and 35 nucleotides long is preferred, with an address tag portion 15 to 20 nucleotides long being most preferred.

6. Promoter Portion

The promoter portion corresponds to the sequence of an RNA polymerase promoter. A promoter portion can be included in an open circle probe so that transcripts can be generated from TS-DNA. The sequence of any promoter may be used, but simple promoters for RNA polymerases without complex requirements are preferred. It is also preferred that the promoter is not recognized by any RNA polymerase that may be present in the sample containing the target nucleic acid sequence. Preferably, the promoter portion corresponds to the sequence of a T7 or SP6 RNA polymerase promoter. The T7 and SP6 RNA polymerases are highly specific for particular promoter sequences. Other promoter sequences specific for RNA polymerases with this characteristic would also be preferred. Because promoter sequences are generally recognized by specific RNA polymerases, the cognate polymerase for the promoter portion of the OCP should be used for transcriptional amplification. Numerous promoter sequences are known and any promoter specific for a suitable RNA polymerase can be used. The promoter portion can be located anywhere within the spacer region of an OCP and can be in either orientation. Preferably, the promoter portion is immediately adjacent to the left target probe and is oriented to promote transcription toward the 3' end of the open circle probe. This orientation results in transcripts that are complementary to

TS-DNA, allowing independent detection of TS-DNA and the transcripts, and prevents transcription from interfering with rolling circle replication.

D. Gap Oligonucleotides

Gap oligonucleotides are oligonucleotides that are complementary to all or a part of that portion of a target sequence which covers a gap space between the ends of a hybridized open circle probe or between hybridized probe/primers and half probes. An example of a gap oligonucleotide and its relationship to a target sequence and open circle probe is shown in Figure 4. Gap oligonucleotides have a phosphate group at their 5' ends and a hydroxyl group at their 3' ends. This facilitates ligation of gap oligonucleotides to open circle probes, or to other gap oligonucleotides. The gap space between the ends of a hybridized open circle probe can be filled with a single gap oligonucleotide, or it can be filled with multiple gap oligonucleotides. For example, two 3 nucleotide gap oligonucleotides can be used to fill a six nucleotide gap space, or a three nucleotide gap oligonucleotide and a four nucleotide gap oligonucleotide can be used to fill a seven nucleotide gap space. Gap oligonucleotides are particularly useful for distinguishing between closely related target sequences. For example, multiple gap oligonucleotides can be used to amplify different allelic variants of a target sequence. By placing the region of the target sequence in which the variation occurs in the gap space formed by an open circle probe, a single open circle probe can be used to amplify each of the individual variants by using an appropriate set of gap oligonucleotides.

E. Detection Labels

To aid in detection and quantitation of nucleic acids amplified using RCA and RCT, detection labels can be directly incorporated into amplified nucleic acids or can be coupled to detection molecules. As used herein, a detection label is any molecule that can be associated with amplified nucleic acid, directly or indirectly, and which results in a measurable, detectable signal, either directly or indirectly. Many such labels for incorporation into nucleic acids or coupling to nucleic acid or antibody probes are known to those of skill in the art. Examples of detection labels suitable for use in RCA and RCT are radioactive isotopes, fluorescent molecules, phosphorescent molecules, enzymes, antibodies, and ligands.

Examples of suitable fluorescent labels include fluorescein (FITC), 5,6-carboxymethyl fluorescein, Texas red, nitrobenz-2-oxa-1,3-diazol-4-yl (NBD), coumarin, dansyl chloride, rhodamine, 4'-6-diamidino-2-phenylinodole (DAPI), and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5 and Cy7. Preferred fluorescent labels are fluorescein (5-carboxyfluorescein-N-hydroxysuccinimide ester) and rhodamine (5,6-tetramethyl rhodamine). Preferred fluorescent labels for combinatorial multicolor coding are FITC and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5 and Cy7. The absorption and emission maxima, respectively, for these fluors are: FITC (490 nm; 520 nm), Cy3 (554 nm; 568 nm), Cy3.5 (581 nm; 588 nm), Cy5 (652 nm: 672 nm), Cy5.5 (682 nm; 703 nm) and Cy7 (755 nm; 778 nm), thus allowing their simultaneous detection. The fluorescent labels can be obtained from a variety of commercial sources, including Molecular Probes, Eugene, OR and Research Organics, Cleveland, Ohio.

Labeled nucleotides are a preferred form of detection label since they can be directly incorporated into the products of RCA and RCT during synthesis. Examples of detection labels that can be incorporated into amplified DNA or RNA include nucleotide analogs such as BrdUrd (Hoy and Schimke, *Mutation Research* **290**:217-230 (1993)), BrUTP (Wansick *et al.*, *J. Cell Biology* **122**:283-293 (1993)) and nucleotides modified with biotin (Langer *et al., Proc. Natl. Acad. Sci. USA* **78**:6633 (1981)) or with suitable haptens such as digoxygenin (Kerkhof, *Anal. Biochem.* **205**:359-364 (1992)). Suitable fluorescence-labeled nucleotides are Fluorescein-isothiocyanate-dUTP, Cyanine-3-dUTP and Cyanine-5-dUTP (Yu *et al., Nucleic Acids Res.*, **22**:3226-3232 (1994)). A preferred nucleotide analog detection label for DNA is BrdUrd (BUDR triphosphate, Sigma), and a preferred nucleotide analog detection label for RNA is Biotin-16-uridine-5'-triphosphate (Biotin-16-dUTP, Boehringher Mannheim). Fluorescein, Cy3, and Cy5 can be linked to dUTP for direct labeling. Cy3.5 and Cy7 are available as avidin or anti-digoxygenin conjugates for secondary detection of biotin- or digoxygenin-labeled probes.

Detection labels that are incorporated into amplified nucleic acid, such as biotin, can be subsequently detected using sensitive methods well-known in the art. For example, biotin can be detected using streptavidin-alkaline phosphatase conjugate (Tropix, Inc.), which is bound to the biotin and subsequently detected by

chemiluminescence of suitable substrates (for example, chemiluminescent substrate CSPD: disodium, 3-(4-methoxyspiro-[1,2,-dioxetane-3-2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}]decane]-4-yl) phenyl phosphate; Tropix, Inc.).

A preferred detection label for use in detection of amplified RNA is acridinium-ester-labeled DNA probe (GenProbe, Inc., as described by Arnold *et al., Clinical Chemistry* **35**:1588-1594 (1989)). An acridinium-ester-labeled detection probe permits the detection of amplified RNA without washing because unhybridized probe can be destroyed with alkali (Arnold *et al.* (1989)).

Molecules that combine two or more of these detection labels are also considered detection labels. Any of the known detection labels can be used with the disclosed probes, tags, and method to label and detect nucleic acid amplified using the disclosed method. Methods for detecting and measuring signals generated by detection labels are also known to those of skill in the art. For example, radioactive isotopes can be detected by scintillation counting or direct visualization; fluorescent molecules can be detected with fluorescent spectrophotometers; phosphorescent molecules can be detected with a spectrophotometer or directly visualized with a camera; enzymes can be detected by detection or visualization of the product of a reaction catalyzed by the enzyme; antibodies can be detected by detecting a secondary detection label coupled to the antibody. Such methods can be used directly in the disclosed method of amplification and detection. As used herein, detection molecules are molecules which interact with amplified nucleic acid and to which one or more detection labels are coupled.

F. Detection Probes

Detection probes are labeled oligonucleotides having sequence complementary to detection tags on TS-DNA or transcripts of TS-DNA. The complementary portion of a detection probe can be any length that supports specific and stable hybridization between the detection probe and the detection tag. For this purpose, a length of 10 to 35 nucleotides is preferred, with a complementary portion of a detection probe 16 to 20 nucleotides long being most preferred. Detection probes can contain any of the detection labels described above. Preferred labels are biotin and fluorescent molecules. A particularly preferred detection probe is a molecular beacon. Molecular beacons are detection

probes labeled with fluorescent moieties where the fluorescent moieties fluoresce only when the detection probe is hybridized (Tyagi and Kramer, *Nature Biotechnology* **14**:303-308 (1996)). The use of such probes eliminates the need for removal of unhybridized probes prior to label detection because the unhybridized detection probes will not produce a signal. This is especially useful in multiplex assays.

A preferred form of detection probe, referred to herein as a collapsing detection probe, contains two separate complementary portions, a ligand, or both. The presence of two complementary portions allows each detection probe to hybridize to two detection tags in TS-DNA. In this way, the detection probe forms a bridge between different parts of the TS-DNA. The combined action of numerous collapsing detection probes hybridizing to TS-DNA will be to form a collapsed network of cross-linked TS-DNA. Collapsed TS-DNA occupies a much smaller volume than free, extended TS-DNA, and includes whatever detection label is present on the detection probe. This result is a compact and discrete detectable signal for each TS-DNA. Collapsing TS-DNA is useful both for in situ hybridization applications and for multiplex detection because it allows detectable signals to be spatially separate even when closely packed. Collapsing TS-DNA is especially preferred for use with combinatorial multicolor coding.

TS-DNA collapse can also be accomplished through the use of ligand/ligand binding pairs (such as biotin and avidin) or hapten/antibody pairs. For this purpose, ligands can be incorporated into TS-DNA or associated with TS-DNA by hybridization of collapsing detection probes containing ligands. A nucleotide analog, BUDR, can be incorporated into TS-DNA during rolling circle replication. Collapsing detection probes containing a ligand can be used to collapse TS-DNA. When biotinylated antibodies specific for BUDR and avidin are added, a cross-linked network of TS-DNA forms, bridged by avidin-biotin-antibody conjugates, and the TS-DNA collapses into a compact structure. Collapsing detection probes and biotin-mediated collapse can also be used together to collapse TS-DNA.

G. Address Probes

An address probe is an oligonucleotide having a sequence complementary to address tags on TS-DNA or transcripts of TS-DNA. The complementary

portion of an address probe can be any length that supports specific and stable hybridization between the address probe and the address tag. For this purpose, a length of 10 to 35 nucleotides is preferred, with a complementary portion of an address probe 12 to 18 nucleotides long being most preferred. Preferably, the complementary portion of an address probe is complementary to all or a portion of the target probe portions of an OCP. Most preferably, the complementary portion of an address probe is complementary to a portion of either or both of the left and right target probe portions of an OCP and all or a part of any gap oligonucleotides or gap sequence created in a gap-filling operation (see Figure 1, Figure 4). Address probe can contain a single complementary portion or multiple complementary portions. Preferably, address probes are coupled, either directly or via a spacer molecule, to a solid-state support. Such a combination of address probe and solid-state support are a form of solid-state detector.

H. DNA Strand Displacement Primers

Primers used for secondary DNA strand displacement are referred to herein as DNA strand displacement primers. One form of DNA strand displacement primer, referred to herein as a secondary DNA strand displacement primer, is an oligonucleotide having sequence matching part of the sequence of an OCP or ATC. This sequence is referred to as the matching portion of the secondary DNA strand displacement primer. This matching portion of a secondary DNA strand displacement primer is complementary to sequences in TS-DNA. The matching portion of a secondary DNA strand displacement primer may be complementary to any sequence in TS-DNA. However, it is preferred that it not be complementary TS-DNA sequence matching either the rolling circle replication primer or a tertiary DNA strand displacement primer, if one is being used. This prevents hybridization of the primers to each other. The matching portion of a secondary DNA strand displacement primer may be complementary to all or a portion of the target sequence. In this case, it is preferred that the 3' end nucleotides of the secondary DNA strand displacement primer are complementary to the gap sequence in the target sequence. It is most preferred that nucleotide at the 3' end of the secondary DNA strand displacement primer falls complementary to the last nucleotide in the gap sequence of the target sequence, that is, the 5' nucleotide in the gap sequence of the target sequence. The matching portion of a secondary DNA strand

displacement primer can be any length that supports specific and stable hybridization between the primer and its complement. Generally this is 12 to 35 nucleotides long, but is preferably 18 to 25 nucleotides long.

It is preferred that secondary DNA strand displacement primers also contain additional sequence at their 5' end that does not match any part of the OCP or ATC. This sequence is referred to as the non-matching portion of the secondary DNA strand displacement primer. The non-matching portion of the secondary DNA strand displacement primer, if present, serves to facilitate strand displacement during DNA replication. The non-matching portion of a secondary DNA strand displacement primer may be any length, but is generally 1 to 100 nucleotides long, and preferably 4 to 8 nucleotides long.

Another form of DNA strand displacement primer, referred to herein as a tertiary DNA strand displacement primer, is an oligonucleotide having sequence complementary to part of the sequence of an OCP or ATC. This sequence is referred to as the complementary portion of the tertiary DNA strand displacement primer. This complementary portion of the tertiary DNA strand displacement primer matches sequences in TS-DNA. The complementary portion of a tertiary DNA strand displacement primer may be complementary to any sequence in the OCP or ATC. However, it is preferred that it not be complementary OCP or ATC sequence matching the secondary DNA strand displacement primer. This prevents hybridization of the primers to each other. Preferably, the complementary portion of the tertiary DNA strand displacement primer has sequence complementary to a portion of the spacer portion of an OCP. The complementary portion of a tertiary DNA strand displacement primer can be any length that supports specific and stable hybridization between the primer and its complement. Generally this is 12 to 35 nucleotides long, but is preferably 18 to 25 nucleotides long. It is preferred that tertiary DNA strand displacement primers also contain additional sequence at their 5' end that is not complementary to any part of the OCP or ATC. This sequence is referred to as the non-complementary portion of the tertiary DNA strand displacement primer. The non-complementary portion of the tertiary DNA strand displacement primer, if present, serves to facilitate strand displacement during DNA replication. The non-complementary portion of a tertiary DNA strand displacement primer may be any length, but is generally 1 to 100 nucleotides long,

and preferably 4 to 8 nucleotides long. A rolling circle replication primer is a preferred form of tertiary DNA strand displacement primer.

DNA strand displacement primers may also include modified nucleotides to make them resistant to exonuclease digestion. For example, the primer can have three or four phosphorothioate linkages between nucleotides at the 5' end of the primer. Such nuclease resistant primers allow selective degradation of excess unligated OCP and gap oligonucleotides that might otherwise interfere with hybridization of detection probes, address probes, and secondary OCPs to the amplified nucleic acid. DNA strand displacement primers can be used for secondary DNA strand displacement and strand displacement cascade amplification, both described below.

I. Oligonucleotide synthesis

Probe/primers, half probes, amplification target circles, open circle probes, gap oligonucleotides, rolling circle replication primers, detection probes, address probes, DNA strand displacement primers, and any other oligonucleotides can be synthesized using established oligonucleotide synthesis methods. Methods to produce or synthesize oligonucleotides are well known. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see for example, Sambrook *et al., Molecular Cloning: A Laboratory Manual,* 2nd Edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method using a Milligen or Beckman System 1Plus DNA synthesizer (for example, Model 8700 automated synthesizer of Milligen-Biosearch, Burlington, MA or ABI Model 380B). Synthetic methods useful for making oligonucleotides are also described by Ikuta *et al., Ann. Rev. Biochem.* **53**:323-356 (1984), (phosphotriester and phosphite-triester methods), and Narang *et al., Methods Enzymol.*, **65**:610-620 (1980), (phosphotriester method). Protein nucleic acid molecules can be made using known methods such as those described by Nielsen *et al., Bioconjug. Chem.* **5**:3-7 (1994).

Head to head probe/primers (reversed probe/primers) can be made by coupling the 5' ends of a probe oligonucleotide and a primer oligonucleotide using any suitable or known method of coupling. The primer and probe oligonucleotides can be coupled using a linker molecule. It is preferred that such a linker molecule

be made up of nucleotides. It is preferred that head to head probe/primers be synthesized as a single oligonucleotide using first standard phosphoramidite nucleotides for the first portion and then switching to reversed phosphoramidite nucleotides, such as dN-5'-CE phosphoramidites (Glen Research, Sterling, VA), for the second portion. This allows synthesis using standard chemistry in a continuous synthesis operation.

Many of the oligonucleotides described herein are designed to be complementary to certain portions of other oligonucleotides or nucleic acids such that stable hybrids can be formed between them. The stability of these hybrids can be calculated using known methods such as those described in Lesnick and Freier, *Biochemistry* **34**:10807-10815 (1995), McGraw *et al., Biotechniques* **8**:674-678 (1990), and Rychlik *et al., Nucleic Acids Res.* **18**:6409-6412 (1990).

J. Solid-State Detectors

Solid-state detectors are solid-state substrates or supports to which oligonucleotides, such as half probes and rolling circle replication primers, have been coupled. A preferred form of solid-state detector is an array detector. An array detector is a solid-state detector to which multiple different oligonucleotides, such as half probes and rolling circle replication primers, have been coupled in an array, grid, or other organized pattern.

Solid-state substrates for use in solid-state detectors can include any solid material to which oligonucleotides can be coupled. This includes materials such as acrylamide, cellulose, nitrocellulose, glass, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, polysilicates, polycarbonates, teflon, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, polypropylfumerate, collagen, glycosaminoglycans, and polyamino acids. Solid-state substrates can have any useful form including thin films or membranes, beads, bottles, dishes, fibers, woven fibers, shaped polymers, particles and microparticles. A preferred form for a solid-state substrate is a microtiter dish. The most preferred form of microtiter dish is the standard 96-well type.

Half probes and rolling circle replication primers immobilized on a solid-state substrate allow capture of specific target molecules or amplification target circles on a solid-state detector. Such capture provides a convenient means of

washing away reaction components that might interfere with subsequent detection steps. By attaching different half probes to different regions of a solid-state detector, different target molecules can be captured at different, and therefore diagnostic, locations on the solid-state detector. For example, half probes specific for multiple different target sequences can be immobilized on a glass slide, each in a different spot. Ligation of probe/primers and amplification of hybridized ATCs will occur only on those spots corresponding to half probes for which the corresponding target sequences were present in a sample. Similarly, by attaching different rolling circle replication primers to different regions of a solid-state detector, different amplification target circles can be captured at different locations on the solid-state detector. This results in amplification and immobilization of the resulting TS-DNA at different, and therefore diagnostic, locations on the solid-state detector.

Methods for immobilization of oligonucleotides to solid-state substrates are well established. Oligonucleotides, including half probes and rolling circle replication primers, can be coupled to substrates using established coupling methods. For example, suitable attachment methods are described by Pease *et al., Proc. Natl. Acad. Sci. USA* **91**(11):5022-5026 (1994), and Khrapko *et al., Mol Biol (Mosk) (USSR)* 25:718-730 (1991). A method for immobilization of 3'-amine oligonucleotides on casein-coated slides is described by Stimpson *et al., Proc. Natl. Acad. Sci. USA* **92**:6379-6383 (1995). A preferred method of attaching oligonucleotides to solid-state substrates is described by Guo *et al., Nucleic Acids Res.* 22:5456-5465 (1994).

The immobilization and arraying of the half probe molecules or rolling circle replication primers to solid supports can be accomplished using any suitable technique. For example, immobilization can be accomplished either by *in situ* DNA synthesis (Maskos and Southern, *Nucleic Acids Research,* **20**:1679-1684 (1992); Pease *et al., Proc. Natl Acad. Sci. USA,* **91**:5022-5026 (1994)) or by covalent attachment of chemically synthesized oligonucleotides (Guo *et al., Nucleic Acids Research,* **22**:5456-5465 (1994)) in combination with robotics arraying technologies. Other immobilization techniques are described in U.S. Patent No. 5,412,087 to McGall *et al.*, U.S. Patent No. 5,429,807 to Matson *et al.*, and U.S. Patent No. 5,510,270 to Fodor *et al.* Thousands of different half probes

or rolling circle replication primers can be arrayed onto a small area on a solid support to interrogate thousands of target DNA/RNA molecules. When not using randomized probe/primers, it is preferred that up to 120 half probes be used in any array. An array with 120 probes can be easily fit onto an area of one square centimeter on a solid support such as a microscope glass slide with currently available technologies.

K. Solid-State Samples

Solid-state samples are solid-state substrates or supports to which target molecules or target sequences have been coupled or adhered. Target molecules or target sequences are preferably delivered in a target sample or assay sample. Cytological and histological preparations can be considered solid-state samples. A preferred form of solid-state sample is an array sample. An array sample is a solid-state sample to which multiple different target samples or assay samples have been coupled or adhered in an array, grid, or other organized pattern.

Solid-state substrates for use in solid-state samples can include any solid material to which target molecules or target sequences can be coupled or adhered. This includes materials such as acrylamide, cellulose, nitrocellulose, glass, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, polysilicates, polycarbonates, teflon, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, polypropylfumerate, collagen, glycosaminoglycans, and polyamino acids. Solid-state substrates can have any useful form including thin films or membranes, beads, bottles, dishes, slides, fibers, woven fibers, shaped polymers, particles and microparticles. Preferred forms for a solid-state substrate are microtiter dishes and glass slides. The most preferred form of microtiter dish is the standard 96-well type.

Target molecules and target sequences immobilized on a solid-state substrate allow formation of target-specific TS-DNA localized on the solid-state substrate. Such localization provides a convenient means of washing away reaction components that might interfere with subsequent detection steps, and a convenient way of assaying multiple different samples simultaneously. Diagnostic TS-DNA can be independently formed at each site where a different sample is adhered. For immobilization of target sequences or other oligonucleotide

molecules to form a solid-state sample, the methods described above for solid-state detectors can be used. Where the target molecule is a protein, the protein can be immobilized on a solid-state substrate generally as described above for the immobilization of antibodies.

A preferred form of solid-state substrate is a glass slide to which up to 256 separate target or assay samples have been adhered as an array of small dots. Each dot is preferably from 0.1 to 2.5 mm in diameter, and most preferably around 2.5 mm in diameter. Such microarrays can be fabricated, for example, using the method described by Schena *et al., Science* 270:487-470 (1995). Briefly, microarrays can be fabricated on poly-L-lysine-coated microscope slides (Sigma) with an arraying machine fitted with one printing tip. The tip is loaded with 1μl of a DNA sample (0.5 mg/ml) from, for example, 96-well microtiter plates and deposited approximately 0.005 μl per slide on multiple slides at the desired spacing. The printed slides can then be rehydrated for 2 hours in a humid chamber, snap-dried at 100°C for 1 minute, rinsed in 0.1% SDS, and treated with 0.05% succinic anhydride prepared in buffer consisting of 50% 1-methyl-2-pyrrolidinone and 50% boric acid. The DNA on the slides can then be denatured in, for example, distilled water for 2 minutes at 90°C immediately before use. Microarray solid-state samples can be scanned with, for example, a laser fluorescent scanner with a computer-controlled XY stage and a microscope objective. A mixed gas, multiline laser allows sequential excitation of multiple fluorophores.

L. DNA ligases

Any DNA ligase is suitable for use in the disclosed amplification method. Preferred ligases are those that preferentially form phosphodiester bonds at nicks in double-stranded DNA. That is, ligases that fail to ligate the free ends of single-stranded DNA at a significant rate are preferred. Thermostable ligases are especially preferred. Many suitable ligases are known, such as T4 DNA ligase (Davis *et al., Advanced Bacterial Genetics - A Manual for Genetic Engineering* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1980)), *E. coli* DNA ligase (Panasnko *et al., J. Biol. Chem.* **253**:4590-4592 (1978)), AMPLIGASE® (Kalin *et al., Mutat. Res.*, **283**(2):119-123 (1992); Winn-Deen *et al., Mol Cell Probes* (England) 7(3):179-186 (1993)), Taq DNA ligase (Barany, *Proc. Natl. Acad. Sci. USA* **88**:189-193 (1991), *Thermus thermophilus* DNA ligase (Abbott

Laboratories), *Thermus scotoductus* DNA ligase and *Rhodothermus marinus* DNA ligase (Thorbjarnardottir *et al., Gene* **151**:177-180 (1995)). T4 DNA ligase is preferred for ligations involving RNA target sequences due to its ability to ligate DNA ends involved in DNA:RNA hybrids (Hsuih *et al., Quantitative detection of HCV RNA using novel ligation-dependent polymerase chain reaction,* American Association for the Study of Liver Diseases (Chicago, IL, November 3-7, 1995)).

The frequency of non-target-directed ligation catalyzed by a ligase can be determined as follows. LM-RCA is performed with an open circle probe and a gap oligonucleotide in the presence of a target sequence. Non-targeted-directed ligation products can then be detected by using an address probe specific for the open circle probe ligated without the gap oligonucleotide to capture TS-DNA from such ligated probes. Target directed ligation products can be detected by using an address probe specific for the open circle probe ligated with the gap oligonucleotide. By using a solid-state detector with regions containing each of these address probes, both target-directed and non-target-directed ligation products can be detected and quantitated. The ratio of target-directed and non-target-directed TS-DNA produced provides a measure of the specificity of the ligation operation. Target-directed ligation can also be assessed as discussed in Barany (1991).

M. DNA polymerases

DNA polymerases useful in the rolling circle replication step of RCA must perform rolling circle replication of primed single-stranded circles. Such polymerases are referred to herein as rolling circle DNA polymerases. For rolling circle replication, it is preferred that a DNA polymerase be capable of displacing the strand complementary to the template strand, termed strand displacement, and lack a 5' to 3' exonuclease activity. Strand displacement is necessary to result in synthesis of multiple tandem copies of the ligated OCP. A 5' to 3' exonuclease activity, if present, might result in the destruction of the synthesized strand. It is also preferred that DNA polymerases for use in the disclosed method are highly processive. The suitability of a DNA polymerase for use in the disclosed method can be readily determined by assessing its ability to carry out rolling circle replication. Preferred rolling circle DNA polymerases are bacteriophage ϕ29 DNA polymerase (U.S. Patent Nos. 5,198,543 and 5,001,050 to Blanco *et al.*), phage M2

DNA polymerase (Matsumoto *et al., Gene* **84**:247 (1989)), phage ϕPRD1 DNA polymerase (Jung *et al., Proc. Natl. Acad. Sci. USA* **84**:8287 (1987)), VENT® DNA polymerase (Kong *et al., J. Biol. Chem.* **268**:1965-1975 (1993)), Klenow fragment of DNA polymerase I (Jacobsen *et al., Eur. J. Biochem.* **45**:623-627 (1974)), T5 DNA polymerase (Chatterjee *et al., Gene* **97**:13-19 (1991)), PRD1 DNA polymerase (Zhu and Ito, *Biochim. Biophys. Acta.* **1219**:267-276 (1994)), modified T7 DNA polymerase (Tabor and Richardson, *J. Biol. Chem.* **262**:15330-15333 (1987); Tabor and Richardson, *J. Biol. Chem.* **264**:6447-6458 (1989); Sequenase™ (U.S. Biochemicals)), and T4 DNA polymerase holoenzyme (Kaboord and Benkovic, *Curr. Biol.* **5**:149-157 (1995)). ϕ29 DNA polymerase is most preferred.

Strand displacement can be facilitated through the use of a strand displacement factor, such as helicase. It is considered that any DNA polymerase that can perform rolling circle replication in the presence of a strand displacement factor is suitable for use in the disclosed method, even if the DNA polymerase does not perform rolling circle replication in the absence of such a factor. Strand displacement factors useful in RCA include BMRF1 polymerase accessory subunit (Tsurumi *et al., J. Virology* **67**(12):7648-7653 (1993)), adenovirus DNA-binding protein (Zijderveld and van der Vliet, *J. Virology* **68**(2):1158-1164 (1994)), herpes simplex viral protein ICP8 (Boehmer and Lehman, *J. Virology* **67**(2):711-715 (1993); Skaliter and Lehman, *Proc. Natl. Acad. Sci. USA* **91**(22):10665-10669 (1994)), single-stranded DNA binding proteins (SSB; Rigler and Romano, *J. Biol. Chem.* **270**:8910-8919 (1995)), and calf thymus helicase (Siegel *et al., J. Biol. Chem.* **267**:13629-13635 (1992)).

The ability of a polymerase to carry out rolling circle replication can be determined by using the polymerase in a rolling circle replication assay such as those described in Fire and Xu, *Proc. Natl. Acad. Sci. USA* 92:4641-4645 (1995) and in Example 1.

Another type of DNA polymerase can be used if a gap-filling synthesis step is used, such as in gap-filling LM-RCA. When using a DNA polymerase to fill gaps, strand displacement by the DNA polymerase is undesirable. Such DNA polymerases are referred to herein as gap-filling DNA polymerases. Unless otherwise indicated, a DNA polymerase referred to herein without specifying it as

a rolling circle DNA polymerase or a gap-filling DNA polymerase, is understood to be a rolling circle DNA polymerase and not a gap-filling DNA polymerase. Preferred gap-filling DNA polymerases are T7 DNA polymerase (Studier *et al., Methods Enzymol.* **185**:60-89 (1990)), DEEP VENT® DNA polymerase (New England Biolabs, Beverly, MA), modified T7 DNA polymerase (Tabor and Richardson, *J. Biol. Chem.* **262**:15330-15333 (1987); Tabor and Richardson, *J. Biol. Chem.* **264**:6447-6458 (1989); Sequenase™ (U.S. Biochemicals)), and T4 DNA polymerase (Kunkel *et al., Methods Enzymol.* **154**:367-382 (1987)). An especially preferred type of gap-filling DNA polymerase is the *Thermus flavus* DNA polymerase (MBR, Milwaukee, WI). The most preferred gap-filling DNA polymerase is the Stoffel fragment of Taq DNA polymerase (Lawyer *et al., PCR Methods Appl.* **2**(4):275-287 (1993), King *et al., J. Biol. Chem.* **269**(18):13061-13064 (1994)).

The ability of a polymerase to fill gaps can be determined by performing gap-filling LM-RCA. Gap-filling LM-RCA is performed with an open circle probe that forms a gap space when hybridized to the target sequence. Ligation can only occur when the gap space is filled by the DNA polymerase. If gap-filling occurs, TS-DNA can be detected, otherwise it can be concluded that the DNA polymerase, or the reaction conditions, is not useful as a gap-filling DNA polymerase.

N. Other Materials

Other materials may be used with certain embodiments of the disclosed method. These include interrogation probes, degenerate probes, interrogation primers, caged oligonucleotides, reporter binding agents, and peptide nucleic acid clamps, all of which are described in PCT Application WO 97/19193. An interrogation probe is an oligonucleotide having a sequence complementary to portions of TS-DNA or transcripts of TS-DNA. Interrogation probes are intended for use in primer extension sequencing operations following rolling circle amplification of an OCP or amplification target circle. Degenerate probes are oligonucleotides intended for use in primer extension sequencing operations following rolling circle amplification of an OCP or amplification target circle. Degenerate probes are combined with interrogation probes to form interrogation primers. An interrogation primer is an oligonucleotide having a sequence

complementary to portions of TS-DNA or transcripts of TS-DNA. Caged oligonucleotides are oligonucleotides having a caged nucleotide at their 3' end. The cage structure is a removable blocking group which prevents the 3' hydroxyl from participating in nucleotide addition and ligation reactions. A reporter binding agent is a specific binding molecule coupled or tethered to an oligonucleotide. The specific binding molecule is referred to as the affinity portion of the reporter binding agent and the oligonucleotide is referred to as the oligonucleotide portion of the reporter binding agent. As used herein, a specific binding molecule is a molecule that interacts specifically with a particular molecule or moiety. Any RNA polymerase which can carry out transcription *in vitro* and for which promoter sequences have been identified can be used in the disclosed rolling circle transcription method. Peptide nucleic acid (PNA) clamps are peptide nucleic acids complementary to sequences in both the left target probe portion and right target probe portion of an OCP, but not to the sequence of any gap oligonucleotides or filled gap space in the ligated OCP. Thus, a PNA clamp can hybridize only to the ligated junction of OCPs that have been illegitimately ligated, that is, ligated in a non-target-directed manner.

The materials described above can be packaged together in any suitable combination as a kit useful for performing the disclosed method.

II. Method

The disclosed rolling circle amplification (RCA) method involves replication of circular single-stranded DNA molecules. In RCA, a rolling circle replication primer hybridizes to circular OCP or ATC molecules followed by rolling circle replication of the OCP or ATC molecules using a strand-displacing DNA polymerase. Amplification takes place during rolling circle replication in a single reaction cycle. Rolling circle replication results in large DNA molecules containing tandem repeats of the OCP or ATC sequence. This DNA molecule is referred to as a tandem sequence DNA (TS-DNA).

In one form of the method, referred to as bipartite primer rolling circle amplification (BP-RCA), RCA of the amplification target circle (ATC) depends on the formation of a primer by target-mediated ligation. In the presence of a nucleic acid molecule having the target sequence, a probe and a combination probe/primer oligonucleotide can hybridize to adjacent sites on the target sequence allowing the

probes to be ligated together. By attaching the first probe to a substrate such as a bead or glass slide, unligated probe/primer can be removed after ligation. The only primers remaining will be primers ligated, via the probe portion of the probe/primer, to the first probe. The ligated primer can then be used to prime replication of its cognate ATC. In this way, an ATC will only be replicated if the target sequence (to which its cognate probe/primer is complementary) is present.

BP-RCA is useful, for example, for determining which target sequences are present in a nucleic acid sample, or for determining which samples contain a target sequence. The former can be accomplished, for example, by using a variety of probe sequences, each complementary to a different target sequence of interest, and different ATCs designed to be primed by only one of the primer sequences (present in a probe/primer). Probe/primers complementary to a given target sequence of interest will only be ligated to the immobilized first probe when that target sequence is present in the nucleic acid sample, and only those ATCs complementary to ligated primers will be amplified. As a result, only those ATCs corresponding to target sequences in the nucleic acid sample will be amplified, thus identifying the target sequences present.

Determining which samples contain a target sequence can be accomplished, for example, by using multiple copies of a single form of probe complementary to the target sequence of interest and multiple copies of a single form of ATC designed to be primed by the primer sequence (present in the probe/primer). Parallel assays can then be performed, each using the same probe, probe/primer, and ATC, where each assay uses a different sample. This can be accomplished, for example, by coating a glass slide with the probe, spotting the probe/primer in an array of spots on the slide, spotting a different sample on each of the array spots, and ligating. If a sample contains the target sequence of interest, the probe/primer will be ligated to the immobilized first probe in that assay spot and the ATC, added after washing away unligated probe/primer, will be amplified in assay spots where the target sequence was present.

The amplification of small circularized oligonucleotides by BP-RCA is rapid, technically simple, and the amplified DNA will not diffuse away from the site of synthesis. RCA products may be detected by incorporating haptens or fluors directly, and it is possible to use circles with biased base compositions to obtain

differential labeling. A larger range of labeling combinations is attainable by collapsing the amplified DNA. For DNA microarray applications, one may use any two-color system which permits measurements of relative allele frequencies by single molecule counting. The two-circle/two-primer signal generating system shown in Figure 2 and described in Example 3 is extensible to any number of arrayed probes, while retaining the use of the same pair of amplification target circles. Two different allele-discriminating primers should be used for each mutational locus being assayed.

While photolithographic DNA microarray technology enables massively parallel assays for mutation detection, altered bases are only detectable using prior methods of detection if they constitute a sizable fraction of the DNA population. Thus, such arrays are well suited for the detection of germline mutations, but not rare somatic mutations when using prior methods of detection. BP-RCA extends the utility of DNA microarrays by permitting the detection of infrequent mutations in the presence of an excess of wild-type DNA. The detection limit for such mutations will be determined by the stringency of the DNA ligation reaction, which should be improved by using new ligase variants (Luo *et al., Nucl. Acids Res.* **24**:3071-3078 (1996)).

BP-RCA is also useful for mRNA profiling, where standard cDNA hybridization approaches may not be sensitive enough to detect changes in the concentration of low abundance gene products. The single molecule counting approach is both sensitive and linear in its response to target concentration. BP-RCA can also be used with a multiparametric color coding such as Combinatorial Multicolor Coding (Speicher *et al., Nature Genet.* **12**:368-375 (1996)). For example, a 6-fluor tagging approach would permit the simultaneous identification of 63 different types of signals on a surface by virtue of their unique spectral signatures, enabling powerful multiplex BP-RCA assays.

BP-RCA can be carried out as follows. This example is illustrated in Figure 2.

1. A target DNA molecule (T) is hybridized under stringent hybridization conditions to a half probe molecule (P1) immobilized on a solid support (S). The half probe molecule (P1) is an oligodeoxyribonucleotide which can hybridize specifically to a target sequence in the target molecule. The target molecule can be

on any type and from any source. The half probe is immobilized on the solid support with 5' end up. This orientation is preferred because that will eliminate the possibility of the nonspecific priming by the 3' end of the half probe to the amplification target circle in the rolling circle amplification (step 6).

2. A probe/primer molecule is hybridized to the target molecule. The probe/primer includes a target probe portion (P2), which hybridizes to the target sequence, and a primer portion (Pr). The probe/primer is designed in such a way that it hybridizes to the region of the target sequence adjacent to the half probe with its 3' end next to the 5' end of the half probe. The primer portion of the probe/primer must provide a free 3' end that will later serve as a primer for amplification of the ATC. Therefore, the covalently coupled target probe portion and primer portion of the probe/primer is an unusual chimeric molecule. It consists of two pieces of oligonucleotides which are covalently coupled together at their 5' ends, thus it contains two free 3' ends.

3. The probe/primer is covalently coupled to the half probe by ligation. The ligation can be accomplished, for example, either enzymatically with DNA ligase (Luo *et al.*, *Nucleic Acids Research,* **24**:3071-3078 (1996)) or chemically (Prakash and Kool, *J. Amer. Chem. Soc.,* **114**:3523-3527 (1992)).

4. The target molecule and any other molecules that are not covalently coupled to the solid support are washed away from the solid surface.

5. A single-stranded circular oligonucleotide template (CT; amplification target circle) is hybridized to the primer portion (Pr) attached to the target probe portion (P2) of the probe/primer.

6. The primer, which functions as a rolling circle replication primer, is extended by strand displacement rolling circle amplification (RCA) using the amplification target circle as a template to produce a linear amplification product having tandem repeats (WO 97/19193 by Yale University).

7. The linear amplified product, referred to as tandem sequence DNA (TS-DNA) is detected. TS-DNA can be detected using any desired nucleic acid detection technique. It is preferred that the TS-DNA be collapsed prior to detection. Collapse and detection can be achieved by many methods. For example, biotinylated deoxyribonucleoside triphosphates can be used in the RCA reaction to incorporate biotin into the amplified linear product and the linear

product is collapsed with avidin or streptavidin. Fluorescence-labeled deoxyribonucleoside triphosphates can also be incorporated into the TS-DNA so that the product can be detected by fluorescence measurement. In another example, fluorescence-labeled and biotinylated oligonucleotide or protein nucleic acid (PNA) can be used to hybridize to the TS-DNA, then the product can be collapsed with avidin/streptavidin and detected by fluorescence measurement.

If the same primer portion is coupled to different target probes, only one amplification target circle is required for the rolling circle amplification of all the probe/primer molecules. Each event of hybridization of a target molecule to the immobilized half probe, ligation of the probe/primer to the half probe, and amplification of the ATC primed by the ligated probe/primer will result in one immobilized amplification product (TS-DNA). As a result, target molecules can be interrogated quantitatively by single molecule counting; that is, by counting single TS-DNA molecules.

One useful application of BP-RCA is the detection of somatic mutations in small needle aspirations or small, heterogeneous tissue samples suspect of harboring a small number of cancer cells or highly mutated hyperplastic cells. BP-RCA can be used for the assessment of somatic mutations at pre-defined positions in genomic DNA using low-density arrays containing 100 to 5000 different specific oligonucleotide probes. Allele discrimination is preferably performed using oligonucleotide ligation with a mutant *Thermus thermophilus* DNA ligase (Luo *et al.*, *Nucl. Acids Res.* **24**:3071-3078 (1996)) which has been reported to provide a specific base discrimination ratio of 1:10,000. Ligation events are detected on a molecule-by-molecule basis using BP-RCA. A single BP-RCA assay using an array of 500 gene targets can generate up to 3.0×10^6 simultaneous base detection measurements using a single glass slide, at a cost of $3.00 per slide (estimate for the cost of bound oligonucleotides). BP-RCA can be used to detect a single somatic base mutation event in a background of even 10,000 wild-type DNA strands.

In another form of the method, referred to as immobilized primer rolling circle amplification (IP-RCA), RCA of the ATC depends on incorporation of a target sequence in the ATC during its formation. If the target sequence has been incorporated, a primer that can hybridize to the sequence will prime RCA of the

ATC. This form of the method is useful for determining which form or forms of a variable sequence are present in a nucleic acid sample. For example, if a particular gene can have one of three sequences in a particular location (such as one wild-type sequence and two different mutant sequences), IP-RCA can be used to incorporate the critical region into an ATC followed by RCA of the ATC using one of three primers, each specific for one of the possible sequences. The ATC will only be replicated if it contains the target sequence complementary to the specific primer.

IP-RCA is useful, for example, for determining which target sequences are present in a nucleic acid sample. This can be accomplished, for example, by using a variety of primer sequences, each complementary to a different target sequence of interest, and different open circle probes (OCPs) designed to form ATCs that can be primed by only one of the primer sequences. Priming will occur only if the proper target sequence is incorporated into the ATC during formation. A mixture of the OCPs designed for the various target sequences can first be mixed with a nucleic acid sample and then subjected to target-mediated, gap-filling ligation. This results in the formation of ATCs with sequences related to target sequences of interest that are present in the nucleic acid sample. The ATCs can then be spread over a slide containing an array of immobilized primers, each primer specific for a different target sequence, and an amplification reaction can be performed. ATCs will be amplified only at spots containing the primer corresponding to that ATC. Thus, the location of amplified products identifies which target sequences are present in the sample.

IP-RCA can be carried out as follows.

An Open Circle Probe (OCP) specific for a target sequence, is designed so as to leave a gap that is anywhere between 1 and 1000, nucleotides in length, which is intended to be filled by a DNA polymerase extension reaction. The OCP can also be designed so as to contain at least one site for a restriction enzyme, preferably in the spacer region that is not part of the target probe portion.

DNA extracted from cells, tissues, or any biological sample is mixed with a mixture of several OCPs (the number of different OCPs may range from 10 to 100, or more) in order to allow both target probe portions of the OCP to hybridize specifically to the intended target sequence. Then an extension-ligation reaction is

carried out with two enzymes, a DNA polymerase and a DNA ligase, using the conditions described by Abravaya *et al.*, 1995 (Nucleic Acids Research, Vol. 23:675-682). The hybridized 3'-end of the hybridized Open Circle Probe acts as the extension primer. The extension reaction catalyzed by DNA polymerase may fill-in anywhere between 1 and 1000, nucleotides. After the gap is filled, it is immediately ligated by DNA ligase, completing the covalent closure of the probe.

The covalently closed probe now contains a copy of the sequence of the target, which was incorporated into the DNA circle by the DNA polymerase. The mutation-detection method consists of combining the gap-filling ligation reaction described above with an ordered oligonucleotide array containing specific primers, and an RCA detection reaction.

A detection array is prepared, comprising 10 to 100 unit cells, or more, where each unit cell contains a high surface concentration of a specific rolling circle replication primer molecule which is an oligodeoxyribonucleotide designed to hybridize specifically to a closed circle of unique sequence, formed by gap-filling and ligation of a specific OCP (an OCP circularized on its specific DNA target, as described above). The rolling circle replication primer is immobilized on the solid support via covalent linkage of its 5' end, and the free 3' terminus is available for hybridization and priming reactions.

The mixture of circularized open circle probes is contacted with the array in a suitable hybridization solution, and the material is incubated to permit binding of the immobilized primers to complementary circular DNAs. The 3' end of each immobilized rolling circle replication primer is complementary to a segment of DNA sequence proximal to the 3'-terminal probe sequence of the original OCP. In the most preferred embodiment, the 3'-terminal portion of the primer (3 to 12 bases) is complementary to an equivalent length of DNA that was copied during the gap-filling ligation reaction. After binding, the primer will be competent for initiating a linear RCA reaction, as shown below:

After hybridization, DNA polymerase (with suitable cofactors, if applicable) is added, in the presence of all four deoxynucleoside triphosphates, and the primer is extended by strand displacement rolling circle amplification (RCA) using a circular oligonucleotide molecule as a template. The TS-DNA generated in this reaction contains multiple copies of the DNA sequence that was copied during

the gap-filling ligation reaction, minus the number of bases that were designed in the overlapping primer sequence (the 3 to 12 overlapping bases mentioned above).

The amplified DNA can be detected in any desirable method as described elsewhere herein. In one embodiment, the next step consists of interrogating a single base of nucleotide sequence in the RCA amplified DNA, which remains covalently bound to its primer. This involves sequencing just one base within the gap region that was filled by DNA polymerase during the fill-ligation step. This sequence will be interrogated by using a specific interrogation primer, which will incorporate a single fluorescent ddNTP. The 3'-end of the primer is positioned 1 base upstream of the base that is known to display genetic polymorphism. A specific primer is required for each OCP, that is, 50 probes will require 50 different primers.

Following amplification of an ATC in the disclosed method, the amplified sequences can be detected and quantified using any of the conventional detection systems for nucleic acids such as detection of fluorescent labels, enzyme-linked detection systems, antibody-mediated label detection, and detection of radioactive labels. Major advantages of this method are that the ligation operation can be manipulated to obtain allelic discrimination, the amplification operation is isothermal, and signals are strictly quantitative because the amplification reaction is linear and is catalyzed by a highly processive enzyme.

A. Ligation

A half probe and a probe/primer, optionally in the presence of one or more gap oligonucleotides, is incubated with a sample containing DNA, RNA, or both, under suitable hybridization conditions, and then ligated to form a bipartite primer. The bipartite primer is a form of rolling circle replication primer. The ligation allows subsequent amplification to be dependent on the presence of a target sequence. Suitable ligases for the ligation of bipartite primers are described above. Ligation conditions are generally known. Most ligases require Mg^{++}. There are two main types of ligases, those that are ATP-dependent and those that are NAD-dependent. ATP or NAD, depending on the type of ligase, should be present during ligation.

An open circle probe, optionally in the presence of one or more gap oligonucleotides, is incubated with a sample containing DNA, RNA, or both, under

suitable hybridization conditions, and then ligated to form a covalently closed circle. The ligated open circle probe is a form of amplification target circle. This operation is similar to ligation of padlock probes described by Nilsson *et al.*, *Science*, **265**:2085-2088 (1994). The ligation operation allows subsequent amplification to be dependent on the presence of a target sequence.

The ligase and ligation conditions can be optimized to limit the frequency of ligation of single-stranded termini. Such ligation events do not depend on the presence of a target sequence. In the case of AMPLIGASE®-catalyzed ligation, which takes place at 60°C, it is estimated that no more than 1 in 1,000,000 molecules with single-stranded DNA termini will be ligated. This is based on the level of non-specific amplification seen with this ligase in the ligase chain reaction. Any higher nonspecific ligation frequency would cause enormously high background amplification in the ligase chain reaction. Using this estimate, an approximate frequency for the generation of non-specifically ligated probes with a correctly placed gap oligonucleotide in at the ligation junction can be calculated. Since two ligation events are involved, the frequency of such events using AMPLIGASE® should be the square of 1 in 1,000,000, or 1 in 1×10^{12}. The number of probes used in a typical ligation reaction of 50 µl is 2×10^{12}. Thus, the number of non-specifically ligated circles containing a correct gap oligonucleotide would be expected to be about 2 per reaction.

When RNA is to be detected, it is preferred that a reverse transcription operation be performed to make a DNA target sequence. Alternatively, an RNA target sequence can be detected directly by using a ligase that can perform ligation on a DNA:RNA hybrid substrate. A preferred ligase for this is T4 DNA ligase.

The efficiency for the ligation of a pair of oligonucleotides perfectly hybridized to the target molecule is of many orders of magnitude higher than that of a pair of oligonucleotides hybridized to the target molecule with some mismatched base pairs (Luo *et al.* (1996), Guo *et al.*, *Nature Biotechnology*, **15**:331-335 (1997)). Therefore, optionally, the probes can be designed for allele discrimination by ligation.

1. Gap-Filling Ligation

The gap space formed by an OCP or a probe/primer-half primer pair hybridized to a target sequence may be filled in by a gap-filling DNA polymerase

during the ligation operation. As an alternative, the gap space can be partially bridged by one or more gap oligonucleotides, with the remainder of the gap filled using DNA polymerase. This modified ligation operation is referred to herein as gap-filling ligation and is the preferred form of ligation for OCPs to be used in IP-RCA. The principles and procedure for gap-filling ligation are generally analogous to the filling and ligation performed in gap LCR (Wiedmann *et al., PCR Methods and Applications* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, NY, 1994) pages S51-S64; Abravaya *et al., Nucleic Acids Res.*, **23**(4):675-682 (1995); European Patent Application EP0439182 (1991)). In the case of LM-RCA, the gap-filling ligation operation is substituted for the normal ligation operation. Gap-filling ligation provides a means for discriminating between closely related target sequences. Gap-filling ligation can be accomplished by using a different DNA polymerase, referred to herein as a gap-filling DNA polymerase. Suitable gap-filling DNA polymerases are described above. Alternatively, DNA polymerases in general can be used to fill the gap when a stop base is used. The use of stop bases in the gap-filling operation of LCR is described in European Patent Application EP0439182. The principles of the design of gaps and the ends of flanking probes to be joined, as described in EP0439182, is generally applicable to the design of the gap spaces and the ends of target probe portions described herein.

To prevent interference of the gap-filling DNA polymerase with rolling circle replication, the gap-filling DNA polymerase can be removed by extraction or inactivated with a neutralizing antibody prior to performing rolling circle replication. Such inactivation is analogous to the use of antibodies for blocking Taq DNA polymerase prior to PCR (Kellogg *et al., Biotechniques* **16**(6):1134-1137 (1994)).

Generally, gap-filling LM-RCA can be performed by, in an LM-RCA reaction, (1) using a target sequence with a central region located between a 5' region and a 3' region, and an OCP where neither the left target probe portion of the open circle probe nor the right target probe portion of the open circle probe is complementary to the central region of the target sequence, and (2) mixing gap-filling DNA polymerase with the OCP-target sample mixture.

B. Replication

The rolling circle replication primers formed by specific ligation or immobilized on a solid support prime rolling circle replication of amplification target circles. This reaction requires the addition of two reagents: (a) amplification target circle, which is complementary to the primer portion of the rolling circle replication primer, and (b) a rolling circle DNA polymerase. The DNA polymerase catalyzes primer extension and strand displacement in a processive rolling circle polymerization reaction that proceeds as long as desired, generating a molecule of up to 100,000 nucleotides or larger that contains up to approximately 1000 tandem copies of a sequence complementary to the amplification target circle or open circle probe. A preferred rolling circle DNA polymerase is the DNA polymerase of the bacteriophage ϕ29.

Radioactive, or modified nucleotides such as bromodeoxyuridine triphosphate can be included during rolling circle replication in order to label the DNA generated in the reaction. Alternatively, suitable precursors that provide a binding moiety such as biotinylated nucleotides (Langer *et al.* (1981)) may be included.

Rolling circle amplification can be engineered to produce TS-DNA of different lengths in an assay involving multiple ATCs. This can be useful for extending the number of different targets that can be detected in a single assay. TS-DNA of different lengths can be produced in several ways. In one embodiment, the base composition of the spacer region of different classes of ATC can be designed to be rich in a particular nucleotide. Then a small amount of the dideoxy nucleotide complementary to the enriched nucleotide can be included in the rolling circle amplification reaction. After some amplification, the dideoxy nucleotides will terminate extension of the TS-DNA product of the class of OCP or ATC enriched for the complementary nucleotide. Other OCPs or ATCs will be less likely to be terminated, since they are not enriched for the complementary nucleotide, and will produce longer TS-DNA products, on average.

In another embodiment, two different classes of ATC can be designed with different primer complement portions. These different primer complement portions are designed to be complementary to a different rolling circle replication primer. Then the two different rolling circle replication primers are used together

in a single rolling circle amplification reaction, but with one of the rolling circle replication primers a caged oligonucleotide. The caged rolling circle replication primer will not support rolling circle replication until the cage structure is removed. Thus, the first, uncaged rolling circle replication primer begins amplification of its cognate amplification target circle(s) when the replication operation begins, the second, caged rolling circle replication primer begins amplification of its cognate amplification target circle(s) only after removal of the cage. The amount of TS-DNA produced from each rolling circle replication primer will differ proportionate to the different effective times of replication. Thus, the amount of TS-DNA made using each type of rolling circle replication primer can be controlled using a caged primer. The use of such a caged primer has the advantage that the caged primer can be provided at a sufficient concentration to efficiently initiate rolling circle replication as soon as it is uncaged (rather than at a lower concentration).

C. Modifications And Additional Operations

1. Detection of Amplification Products

Current detection technology makes a second cycle of RCA unnecessary in many cases. Thus, one may proceed to detect the products of the first cycle of RCA directly. Detection may be accomplished by primary labeling or by secondary labeling, as described below.

(a) Primary Labeling

Primary labeling consists of incorporating labeled moieties, such as fluorescent nucleotides, biotinylated nucleotides, digoxygenin-containing nucleotides, or bromodeoxyuridine, during rolling circle replication in RCA, or during transcription in RCT. For example, one may incorporate cyanine dye UTP analogs (Yu *et al.* (1994)) at a frequency of 4 analogs for every 100 nucleotides. A preferred method for detecting nucleic acid amplified *in situ* is to label the DNA during amplification with BrdUrd, followed by binding of the incorporated BUDR with a biotinylated anti-BUDR antibody (Zymed Labs, San Francisco, CA), followed by binding of the biotin moieties with Streptavidin-Peroxidase (Life Sciences, Inc.), and finally development of fluorescence with Fluorescein-tyramide (DuPont de Nemours & Co., Medical Products Dept.).

(b) Secondary Labeling with Detection Probes

Secondary labeling consists of using suitable molecular probes, referred to as detection probes, to detect the amplified DNA or RNA. For example, an open circle may be designed to contain several repeats of a known arbitrary sequence, referred to as detection tags. A secondary hybridization step can be used to bind detection probes to these detection tags. The detection probes may be labeled as described above with, for example, an enzyme, fluorescent moieties, or radioactive isotopes. By using three detection tags per amplification target circle, and four fluorescent moieties per each detection probe, one may obtain a total of twelve fluorescent signals for every amplification target circle repeat in the TS-DNA, yielding a total of 12,000 fluorescent moieties for every amplification target circle that is amplified by RCA.

(c) Multiplexing and Hybridization Array Detection

RCA is easily multiplexed by using sets of different amplification target circles, each set carrying different primer complement portions designed for binding to specific rolling circle replication primers. Only those open circle probes that are able to find their targets will give rise to TS-DNA. The TS-DNA molecules generated by RCA are of high molecular weight and low complexity; the complexity being the length of the open circle probe or amplification target circle. Different TS-DNA can be detected separately using, for example, different detection probes labeled with different detection labels.

(d) Combinatorial Multicolor Coding

A preferred form of multiplex detection involves the use of a combination of labels that either fluoresce at different wavelengths or are colored differently. One of the advantages of fluorescence for the detection of hybridization probes is that several targets can be visualized simultaneously in the same sample. Using a combinatorial strategy, many more targets can be discriminated than the number of spectrally resolvable fluorophores. Combinatorial labeling provides the simplest way to label probes in a multiplex fashion since a probe fluor is either completely absent (-) or present in unit amounts (+); image analysis is thus more amenable to automaton, and a number of experimental artifacts, such as differential photobleaching of the fluors and the effects of changing excitation source power spectrum, are avoided.

The combinations of labels establish a code for identifying different detection probes and, by extension, different target molecules to which those detection probes are associated with. This labeling scheme is referred to as Combinatorial Multicolor Coding (CMC). Such coding is described by Speicher *et al., Nature Genetics* 12:368-375 (1996). Any number of labels, which when combined can be separately detected, can be used for combinatorial multicolor coding. It is preferred that 2, 3, 4, 5, or 6 labels be used in combination. It is most preferred that 6 labels be used. The number of labels used establishes the number of unique label combinations that can be formed according to the formula 2^N-1, where N is the number of labels. According to this formula, 2 labels forms three label combinations, 3 labels forms seven label combinations, 4 labels forms 15 label combinations, 5 labels forms 31 label combinations, and 6 labels forms 63 label combinations.

For combinatorial multicolor coding, a group of different detection probes are used as a set. Each type of detection probe in the set is labeled with a specific and unique combination of fluorescent labels. For those detection probes assigned multiple labels, the labeling can be accomplished by labeling each detection probe molecule with all of the required labels. Alternatively, pools of detection probes of a given type can each be labeled with one of the required labels. By combining the pools, the detection probes will, as a group, contain the combination of labels required for that type of detection probe. This can be illustrated with a simple example. Starting with seven different types of detection probe, each complementary to a different detection tag and designated 1 through 7, unique identification requires three different labels used in seven combinations. Assigning the combinations arbitrarily, one coding scheme is:

Detection probe	1	2	3	4	5	6	7
label A	+			+	+		+
label B		+		+		+	+
label C			+		+	+	+

As can be seen, detection probe 7 must be labeled with three different labels, A, B, and C. This can be accomplished by labels A, B, and C to each individual detection probe 7 molecule. This is the first option described above. Alternatively, three pools of detection probe 7 can be separately labeled, one pool

with label A, one pool with label B, and one pool with label C. In each pool, individual detection molecules are labeled with a single type of label. Mixing the pools results in a solution of detection probe 7 that collectively contains all three labels as required. Labeling of detection probes requiring different numbers of probes can be accomplished in a similar fashion.

Of course, the two types of labeling schemes described above can be combined, resulting in detection probe molecules with multiple labels combined with detection probe molecules of the same type multiply labeled with different labels. This can be illustrated using the example above. Two pools of detection probe type 7 can be separately labeled, one pool with both labels A and B, and one pool with only label C. Mixing the pools results in a solution of detection probe 7 that collectively contains all three labels as required.

Where each detection probe is labeled with a single label, label combinations can also be generated by using OCPs or ATCs with coded combinations of detection tags complementary to the different detection probes. In this scheme, the OCPs or ATCs will contain a combination of detection tags representing the combination of labels required for a specific label code. Using the example above, a set of seven OCPs or ATCs, designated 1 though 7, would contain one, two, or three detection tags, chosen from a set of three detection tag sequences designated dtA, dtB, and dtC. Each detection tag sequence corresponds to one of the labels, A, B, or C, with each label coupled to one of three detection probes, designated dpA, dpB, or dpC, respectively. An example of the resulting coding scheme would be:

OCP or ATC	1	2	3	4	5	6	7
dtA	+			+	+		+
dtB		+		+		+	+
dtC			+		+	+	+

Hybridization could be performed with a pool of all the different labeled detection probes, dpA, dpB, and dpC. The result would be that TS-DNA generated from OCP 7 would hybridize to all three detection probes, thus labeling the TS-DNA with all three labels. In contrast, TS-DNA generated from OCP 4, for example, would hybridize only to detection probes dpA and dpB, thus labeling the

OCP 4-derived TS-DNA with only labels A and B. This method of coding and detection is preferred.

As described above, rolling circle amplification can be engineered to produce TS-DNA of different lengths in an assay involving multiple ligated OCPs or ATCs. The resulting TS-DNA of different lengths can be distinguished simply on the basis of the size of the detection signal they generate. Thus, the same set of detection probes could be used to distinguish two different sets of generated TS-DNA. In this scheme, two different TS-DNAs, each of a different size but assigned the same color code, would be distinguished by the size of the signal produced by the hybridized detection probes. In this way, a total of 126 different targets can be distinguished on a single solid-state sample using a code with 63 combinations, since the signals will come in two flavors, low amplitude and high amplitude. Thus one could, for example, use the low amplitude signal set of 63 probes for detection of oncogene mutations, and the high amplitude signal set of 63 probes for the detection of a tumor suppressor p53 mutations.

Speicher *et al.* describes a set of fluors and corresponding optical filters spaced across the spectral interval 350-770 nm that give a high degree of discrimination between all possible fluor pairs. This fluor set, which is preferred for combinatorial multicolor coding, consists of 4'-6-diamidino-2-phenylinodole (DAPI), fluorescein (FITC), and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5 and Cy7. Any subset of this preferred set can also be used where fewer combinations are required. The absorption and emission maxima, respectively, for these fluors are: DAPI (350 nm; 456 nm), FITC (490 nm; 520 nm), Cy3 (554 nm; 568 nm), Cy3.5 (581 nm; 588 nm), Cy5 (652 nm; 672 nm), Cy5.5 (682 nm; 703 nm) and Cy7 (755 nm; 778 nm). The excitation and emission spectra, extinction coefficients and quantum yield of these fluors are described by Ernst *et al.*, *Cytometry* 10:3-10 (1989), Mujumdar *et al.*, *Cytometry* 10:11-19 (1989), Yu, *Nucleic Acids Res.* 22:3226-3232 (1994), and Waggoner, *Meth. Enzymology* 246:362-373 (1995). These fluors can all be excited with a 75W Xenon arc.

To attain selectivity, filters with bandwidths in the range of 5 to 16 nm are preferred. To increase signal discrimination, the fluors can be both excited and detected at wavelengths far from their spectral maxima. Emission bandwidths can be made as wide as possible. For low-noise detectors, such as cooled CCD

cameras, restricting the excitation bandwidth has little effect on attainable signal to noise ratios. A list of preferred filters for use with the preferred fluor set is listed in Table 1 of Speicher *et al.* It is important to prevent infra-red light emitted by the arc lamp from reaching the detector; CCD chips are extremely sensitive in this region. For this purpose, appropriate IR blocking filters can be inserted in the image path immediately in front of the CCD window to minimize loss of image quality. Image analysis software can then be used to count and analyze the spectral signatures of fluorescent dots.

Discrimination of individual signals in combinatorial multicolor coding can be enhanced by collapsing TS-DNA generated during amplification. As described above, this is preferably accomplished using collapsing detection probes, biotin-antibody conjugates, or a combination of both. A collapsed TS-DNA can occupy a space of no more than 0.3 microns in diameter. Based on this, it is expected that up to a million discrete signals can be detected in a 2.5 mm sample dot. Such discrimination also results in a large dynamic range for quantitative signal detection. For example, where two separate signals are detected in the same sample dot, a ratio of the two signals up to 1:500,000 can be detected. Thus, the relative numbers of different types of signals (such as multicolor codes) can be determined over a wide range. This is expected to allow determination of, for example, whether a particular target sequence is homozygous or heterozygous in a genomic DNA sample, whether a target sequence was inherited or represents a somatic mutation, and the genetic heterogeneity of a genomic DNA sample, such as a tumor sample. In the first case, a homozygous target sequence would produce twice the number of signals of a heterozygous target sequence. In the second case, an inherited target sequence would produce a number of signals equivalent to a homozygous or heterozygous signal (that is, a large number of signals), while a somatic mutation would produce a smaller number of signals depending on the source of the sample. In the third case, the relative number of cells, from which a sample is derived, that have particular target sequences can be determined. The more cells in the sample with a particular target sequence, the larger the signal.

(e) Detecting Groups of Target Sequences

Multiplex RCA assays are particularly useful for detecting mutations in genes where numerous distinct mutations are associated with certain diseases or

where mutations in multiple genes are involved. For example, although the gene responsible for Huntington's chorea has been identified, a wide range of mutations in different parts of the gene occur among affected individuals. The result is that no single test has been devised to detect whether an individual has one or more of the many Huntington's mutations. A single BP-RCA assay can be used to detect the presence of one or more members of a group of any number of target sequences. This can be accomplished, for example, by designing a probe/primer and half probe for each target sequence in the group, where the target probe portions of each pair of probe/primers half probes are different but the sequence of the primer portions and the sequence of the detection tag portions of all the open circle probes are the same. Where randomized probe/primers are used, only the half probes need be specifically designed for the target sequences. All of the half probes are immobilized on the same solid-state substrate, all of the probe/primers are used together, and the same amplification target circle and detection probe are used to produce and detect TS-DNA. If any of the target sequences are present in the target sample, the probe/primer and half primer for that target will be ligated and an amplification target circle will be amplified to form TS-DNA. Since the detection tags on TS-DNA resulting from amplification of any of the ATCs are the same, TS-DNA resulting from ligation of any of the bipartite primers will be detected in that assay. Detection indicates that at least one member of the target sequence group is present in the target sample. This allows detection of a trait associated with multiple target sequences in a single tube or well.

The above scheme can also be used with arbitrarily chosen groups of target sequences in order to screen for a large number of target sequences without having to perform an equally large number of assays. Initial assays can be performed as described above, each using a different group of pairs of probe/primers and half probes designed to hybridize to a different group of target sequences. Additional assays to determine which target sequence is present can then be performed on only those groups that produce TS-DNA. Such group assays can be further nested if desired.

(g) Enzyme-linked Detection

Amplified nucleic acid labeled by incorporation of labeled nucleotides can be detected with established enzyme-linked detection systems. For example,

amplified nucleic acid labeled by incorporation of biotin-16-UTP (Boehringher Mannheim) can be detected as follows. The nucleic acid is immobilized on a solid substrate since the rolling circle replication primer is immobilized. The substrate is washed and contacted with alkaline phosphatase-streptavidin conjugate (Tropix, Inc., Bedford, MA). This enzyme-streptavidin conjugate binds to the biotin moieties on the amplified nucleic acid. The substrate is again washed to remove excess enzyme conjugate and the chemiluminescent substrate CSPD (Tropix, Inc.) is added and covered with a cover slip. The substrate can then be imaged in a Biorad Fluorimager.

(h) Collapse of Nucleic Acids

As described above, TS-DNA or TS-RNA, which are produced as extended nucleic acid molecules, can be collapsed into a compact structure. It is preferred that the nucleic acid to be collapsed is immobilized on a substrate. In the case of BP-RCA and IP-RCA, the TS-DNA is immobilzed since the rolling circle replication primer is immobilized. A preferred means of collapsing nucleic acids is by hybridizing one or more collapsing probes with the nucleic acid to be collapsed. Collapsing probes are oligonucleotides having a plurality of portions each complementary to sequences in the nucleic acid to be collapsed, or oligonucleotides having a portion complementary to sequence in the nucleic acid to be collapsed and one of a ligand/ligand binding pair (such as biotin and avidin) or a hapten/antibody pair. The complementary portions are referred to as complementary portions of the collapsing probe, where each complementary portion is complementary to a sequence in the nucleic acid to be collapsed. The sequences in the nucleic acid to be collapsed are referred to as collapsing target sequences. The complementary portion of a collapsing probe can be any length that supports specific and stable hybridization between the collapsing probe and the collapsing target sequence. For this purpose, a length of 10 to 35 nucleotides is preferred, with a complementary portion of a collapsing probe 16 to 20 nucleotides long being most preferred. It is preferred that at least two of the complementary portions of a collapsing probe be complementary to collapsing target sequences which are separated on the nucleic acid to be collapsed or to collapsing target sequences present in separate nucleic acid molecules. This allows each detection probe to hybridize to at least two separate collapsing target sequences in the nucleic

acid sample. In this way, the collapsing probe forms a bridge between different parts of the nucleic acid to be collapsed. The combined action of numerous collapsing probes hybridizing to the nucleic acid will be to form a collapsed network of cross-linked nucleic acid. Collapsed nucleic acid occupies a much smaller volume than free, extended nucleic acid, and includes whatever detection probe or detection label hybridized to the nucleic acid. This result is a compact and discrete nucleic acid structure which can be more easily detected than extended nucleic acid. Collapsing nucleic acids is useful both for in situ hybridization applications and for multiplex detection because it allows detectable signals to be spatially separate even when closely packed. Collapsing nucleic acids is especially preferred for use with combinatorial multicolor coding.

Collapsing probes can also contain any of the detection labels described above. This allows detection of the collapsed nucleic acid in cases where separate detection probes or other means of detecting the nucleic acid are not employed. Preferred labels are biotin and fluorescent molecules. A particularly preferred detection probe is a molecular beacon. Molecular beacons are detection probes labeled with fluorescent moieties where the fluorescent moieties fluoresce only when the detection probe is hybridized. The use of such probes eliminates the need for removal of unhybridized probes prior to label detection because the unhybridized detection probes will not produce a signal. This is especially useful in multiplex assays.

TS-DNA collapse can also be accomplished through the use of ligand/ligand binding pairs (such as biotin and avidin) or hapten/antibody pairs. A nucleotide analog, BUDR, can be incorporated into TS-DNA during rolling circle replication. When biotinylated antibodies specific for BUDR and avidin are added, a cross-linked network of TS-DNA forms, bridged by avidin-biotin-antibody conjugates, and the TS-DNA collapses into a compact structure. Biotin-derivatized nucleic acid can be formed in many of the common nucleic acid replication operations such as cDNA synthesis, PCR, and other nucleic acid amplification techniques. In most cases, biotin can be incorporated into the synthesized nucleic acid by either incorporation of biotin-derivatized nucleotides or through the use of biotin-derivatized primers. Collapsing probes and biotin-mediated collapse can also be used together to collapse nucleic acids.

2. Nested LM-RCA

After RCA, a round of LM-RCA can be performed on the TS-DNA produced in the first RCA. This new round of LM-RCA is performed with a new open circle probe, referred to as a secondary open circle probe, having target probe portions complementary to a target sequence in the TS-DNA produced in the first round. When such new rounds of LM-RCA are performed, the amplification is referred to herein as nested LM-RCA. Nested LM-RCA is particularly useful for *in situ* hybridization applications of LM-RCA. Preferably, the target probe portions of the secondary OCP are complementary to a secondary target sequence in the spacer sequences of the TS-DNA produced in the first RCA. The complement of this secondary target sequence is present in the spacer portion of the OCP or ATC used in the first RCA. After mixing the secondary OCP with the TS-DNA, ligation and rolling circle amplification proceed as in LM-RCA. Each ligated secondary OCP generates a new TS-DNA. By having, for example, two secondary target sequence portions in the first round OCP, the new round of LM-RCA will yield two secondary TS-DNA molecules for every OCP or ATC repeat in the TS-DNA produced in the first RCA. Thus, the amplification yield of nested LM-RCA is about 2000-fold. The overall amplification using two cycles of RCA is thus 1000 X 2000 = 2,000,000. Nested LM-RCA can follow any DNA replication or transcription operation described herein, such as RCA, LM-RCA, secondary DNA strand displacement, strand displacement cascade amplification, or transcription. Nested LM-RCA is described in WO 97/19193.

3. Secondary DNA strand displacement and Strand Displacement Cascade Amplification

Secondary DNA strand displacement is another way to amplify TS-DNA. Secondary DNA strand displacement is accomplished by hybridizing secondary DNA strand displacement primers to TS-DNA and allowing a DNA polymerase to synthesize DNA from these primed sites. Since a complement of the secondary DNA strand displacement primer occurs in each repeat of the TS-DNA, secondary DNA strand displacement can result in a level of amplification similar to or larger than that obtained in RCT. The product of secondary DNA strand displacement is referred to as secondary tandem sequence DNA or TS-DNA-2. Secondary DNA strand displacement is described in WO 97/19193.

When secondary DNA strand displacement is carried out in the presence of a tertiary DNA strand displacement primer, an exponential amplification of TS-DNA sequences takes place. This special and preferred mode of secondary DNA strand displacement is referred to as strand displacement cascade amplification (SDCA). In SDCA a secondary DNA strand displacement primer primes replication of TS-DNA to form TS-DNA-2, as described above. The tertiary DNA strand displacement primer strand can then hybridize to, and prime replication of, TS-DNA-2 to form TS-DNA-3. Strand displacement of TS-DNA-3 by the adjacent, growing TS-DNA-3 strands makes TS-DNA-3 available for hybridization with secondary DNA strand displacement primer. This results in another round of replication resulting in TS-DNA-4 (which is equivalent to TS-DNA-2). TS-DNA-4, in turn, becomes a template for DNA replication primed by tertiary DNA strand displacement primer. The cascade continues this manner until the reaction stops or reagents become limiting. This reaction amplifies DNA at an almost exponential rate, although kinetics are not truly exponential because there are stochastically distributed priming failures, as well as steric hindrance events related to the large size of the DNA network produced during the reaction. In a preferred mode of SDCA, the rolling circle replication primer serves as the tertiary DNA strand displacement primer, thus eliminating the need for a separate primer. For this mode, the rolling circle replication primer should be used at a concentration sufficiently high to obtain rapid priming on the growing TS-DNA-2 strands. To optimize the efficiency of SDCA, it is preferred that a sufficient concentration of secondary DNA strand displacement primer and tertiary DNA strand displacement primer be used to obtain sufficiently rapid priming of the growing TS-DNA strand to outcompete TS-DNA for binding to its complementary TS-DNA, and, in the case of secondary DNA strand displacement primer, to outcompete any remaining unligated OCPs and gap oligonucleotides that might be present for binding to TS-DNA. In general, this is accomplished when the secondary DNA strand displacement primer and tertiary DNA strand displacement primer are both in very large excess compared to the concentration of single-stranded sites for hybridization of the DNA strand displacement primers on TS-DNA. For example, it is preferred that the secondary DNA strand displacement primer is in excess compared to the concentration of single-stranded secondary DNA strand

displacement primer complement sites on TS-DNA, TS-DNA-3, TS-DNA-5, and so on. In the case of tertiary DNA strand displacement primer, it is preferred that the tertiary DNA strand displacement primer is in excess compared to the concentration of single-stranded tertiary DNA strand displacement primer complement sites on TS-DNA-2, TS-DNA-4, TS-DNA-6, and so on. Such an excess generally results in a primer hybridizing to its complement in TS-DNA before amplified complementary TS-DNA can hybridize. Optimization of primer concentrations can be aided by analysis of hybridization kinetics (Young and Anderson). In a strand displacement cascade amplification, it is preferred that the concentration of both secondary and tertiary DNA strand displacement primers generally be from 500 nM to 5000 nM, and most preferably from 700 nM to 1000 nM.

A modified form of secondary DNA strand displacement results in amplification of TS-DNA and is referred to as opposite strand amplification (OSA). OSA is the same as secondary DNA strand displacement except that a special form of rolling circle replication primer is used that prevents it from hybridizing to TS-DNA-2. This can be accomplished in a number of ways. For example, the rolling circle replication primer can have an affinity tag coupled to its non-complementary portion allowing the rolling circle replication primer to be removed prior to secondary DNA strand displacement. Alternatively, remaining rolling circle replication primer can be crippled following initiation of rolling circle replication. One preferred form of rolling circle replication primer for use in OSA is designed to form a hairpin that contains a stem of perfectly base-paired nucleotides. The stem can contain 5 to 12 base pairs, most preferably 6 to 9 base pairs. Such a hairpin-forming rolling circle replication primer is a poor primer at lower temperature (less than 40°C) because the hairpin structure prevents it from hybridizing to complementary sequences. The stem should involve a sufficient number of nucleotides in the complementary portion of the rolling circle replication primer to interfere with hybridization of the primer to the OCP or ATC. Generally, it is preferred that a stem involve 5 to 24 nucleotides, and most preferably 6 to 18 nucleotides, of the complementary portion of a rolling circle replication primer. A rolling circle replication primer where half of the stem involves nucleotides in the complementary portion of the rolling circle replication

primer and the other half of the stem involves nucleotides in the non-complementary portion of the rolling circle replication primer is most preferred. Such an arrangement eliminates the need for self-complementary regions in the OCP or ATC when using a hairpin-forming rolling circle replication primer.

The DNA generated by secondary DNA strand displacement can be labeled and/or detected using the same labels, labeling methods, and detection methods described for use with TS-DNA. Most of these labels and methods are adaptable for use with nucleic acids in general. A preferred method of labeling the DNA is by incorporation of labeled nucleotides during synthesis.

4. Transcription Following RCA (RCT)

Once TS-DNA is generated using RCA, further amplification can be accomplished by transcribing the TS-DNA from promoters embedded in the TS-DNA. This combined process, referred to as rolling circle replication with transcription (RCT), or ligation mediated rolling circle replication with transcription (LM-RCT), requires that the OCP or ATC from which the TS-DNA is made have a promoter portion in its spacer region. The promoter portion is then amplified along with the rest of the OCP or ATC resulting in a promoter embedded in each tandem repeat of the TS-DNA. Since transcription, like rolling circle amplification, is a process that can go on continuously (with re-initiation), multiple transcripts can be produced from each of the multiple promoters present in the TS-DNA. RCT effectively adds another level of amplification of ligated OCP sequences.

5. Ligation Mediated Rolling Circle Amplification with Combinatorial Multicolor Coding

A preferred form of rolling circle amplification involving multiplex detection is Ligation Mediated Rolling Circle Amplification with Combinatorial Multicolor Coding (LM-RCA-CMC), which is a combination of LM-RCA and CMC, both as described above. In LM-RCA-CMC, open circle probes and corresponding gap oligonucleotides are designed for the detection of a number of distinct target sequences. DNA samples to be tested are incorporated into a solid-state sample, as described above. The solid-state substrate is preferably a glass slide and the solid-state sample preferably incorporates up to 256 individual target or assay samples arranged in dots. Multiple solid-state samples can be used to

either test more individual samples, or to increase the number of distinct target sequences to be detected. In the later case, each solid-state sample has an identical set of sample dots, and LM-RCA will be carried out using a different set of open circle probes and gap oligonucleotides, collectively referred to as a probe set, for each solid-state sample. This allows a large number of individuals and target sequences to be assayed in a single assay. By using up to six different labels, combinatorial multicolor coding allows up to 63 distinct targets to be detected on a single solid-state sample. When using multiple solid-state substrates and performing LM-RCA with a different set of OCPs and gap oligonucleotides for each solid-state substrate, the same labels can be used with each solid-state sample (although differences between OCPs in each set may require the use of different detection probes). For example, 10 replica slides, each with 256 target sample dots, can be subjected to LM-RCA using 10 different sets of OCPs and gap oligonucleotides, where each set is designed for combinatorial multicolor coding of 63 targets. This result in an assay for detection of 630 different target sequences. Where two or more different target sequences are closely spaced in the DNA of the target or assay sample (for example, when multiple closely spaced mutations of the same gene are targets), it is preferred that the OCPs and gap oligonucleotides for each of the closely spaced target sequences be placed in a different probe set. For this purpose, it is considered that target sequences within 20 nucleotides of each other on a DNA molecule in a target or assay sample are closely spaced. It is not required that multiple targets within the same gene be detected with a different probe set. It is merely preferred that closely spaced target sequences, as defined above, be separately probed.

After rolling circle amplification, a cocktail of detection probes is added, where the cocktail contains color combinations that are specific for each OCP. The design and combination of such detection probes for use in combinatorial multicolor coding is described above. It is preferred that the OCPs be designed with combinatorially coded detection tags to allow use of a single set of singly labeled detection probes. It is also preferred that collapsing detection probes be used. As described above, collapsing probes contain two complementary portions. This allows each detection probe to hybridize to two detection tags in TS-DNA. In this way, the detection probe forms a bridge between different parts of the TS-

DNA. The combined action of numerous collapsing detection probes hybridizing to TS-DNA will be to form a collapsed network of cross-linked TS-DNA. Collapsed TS-DNA occupies a much smaller volume than free, extended TS-DNA, and includes whatever detection label present on the detection probe. This result is a compact and discrete detectable signal for each TS-DNA. Probe binding will, upon collapse, trap a unique combination of colors that was designed a priory on the basis of each probe sequence.

As discussed above, rolling circle amplification can be engineered to produce TS-DNA of different lengths for different OCPs. Such products can be distinguished simply on the basis of the size of the detection signal they generate. Thus, the same set of detection probes could be used to distinguish two different sets of generated TS-DNA. In this scheme, two different TS-DNAs, each of a different size class but assigned the same color code, would be distinguished by the size of the signal produced by the hybridized detection probes. In this way, a total of 126 different targets can be distinguished on a single solid-state sample using a code with 63 combinations, since the signals will come in two flavors, low amplitude and high amplitude. Thus one could, for example, use the low amplitude signal set of 63 probes for detection of an oncogene mutations, and the high amplitude signal set of 63 probes for the detection of a tumor suppressor p53 mutations.

6. Reporter Binding Agent Unimolecular Rolling Amplification

Reporter Binding Agent Unimolecular Rolling Amplification (RBAURA) is a form of RCA where a reporter binding agent provides the rolling circle replication primer for amplification of an amplification target circle. In RBAURA, the oligonucleotide portion of the reporter binding agent serves as a rolling circle replication primer. RBAURA allows RCA to produce an amplified signal (that is, TS-DNA) based on association of the reporter binding agent to a target molecule. The specific primer sequence that is a part of the reporter binding agent provides the link between the specific interaction of the reporter binding agent to a target molecule (via the affinity portion of the reporter binding agent) and RCA. In RBAURA, once the reporter binding agent is associated with a target molecule, an amplification target circle is hybridized to the rolling circle replication primer sequence of the reporter binding agent, followed by amplification of the ATC by

RCA. The resulting TS-DNA incorporates the rolling circle replication primer sequence of the reporter binding agent at one end, thus anchoring the TS-DNA to the site of the target molecule. RBAURA is a preferred RCA method for *in situ* detections. For this purpose, it is preferred that the TS-DNA is collapsed using collapsing detection probes, biotin-antibody conjugates, or both, as described above. RBAURA can be performed using any target molecule. Preferred target molecules are nucleic acids, including amplified nucleic acids such as TS-DNA and amplification target circles, antigens and ligands. Peptide Nucleic Acid Probe Unimolecular Rolling Amplification (PNAPURA) and Locked Antibody Unimolecular Rolling Amplification (LAURA), described in WO 97/19193, are preferred forms of RBAURA.

7. Primer Extension Sequencing

Following amplification, the nucleotide sequence of the amplified sequences can be determined either by conventional means or by primer extension sequencing of amplified target sequence. Primer extension sequencing is also referred herein as chain terminating primer extension sequencing. A preferred form of chain terminating primer extension sequencing, referred to herein as single nucleotide primer extension sequencing, involves the addition of a single chain-terminating nucleotide to a primer (no other nucleotides are added). This form of primer extension sequencing allows interrogation (and identification) of the nucleotide immediately adjacent to the region to which the primer is hybridized. Primer extension sequencing is described in WO 97/19193. Two preferred modes of single nucleotide primer extension sequencing are disclosed.

(a) Unimolecular Segment Amplification and Sequencing

Unimolecular Segment Amplification and Sequencing (USA-SEQ) involves interrogation of a single nucleotide in an amplified target sequence by incorporation of a specific and identifiable nucleotide based on the identity of the interrogated nucleotide. In Unimolecular Segment Amplification and Sequencing (USA-SEQ) individual target molecules are amplified by rolling circle amplification. Following amplification, an interrogation primer is hybridized immediately 5' of the base in the target sequence to be interrogated, and a single chain-terminating nucleotide is added to the end of the primer. The identity of the interrogated base determines which nucleotide is added. By using nucleotides with

unique detection signatures (e.g. different fluorescent labels), the identity of the interrogated base can be determined. The interrogation primer can be a pre-formed single molecule or it can be formed by hybridizing one or more interrogation probes to the amplified target sequences and ligating them together to form an interrogation primer.

(b) Degenerate Probe Primer Extension Sequencing

Degenerate probe primer extension sequencing involves sequential addition of degenerate probes to an interrogation primer hybridized to amplified target sequences. Addition of multiple probes is prevented by the presence of a removable blocking group at the 3' end. After addition of the degenerate probes, the blocking group is removed and further degenerate probes can be added or, as the final operation, the nucleotide next to the end of the interrogation probe, or the last added degenerate probe, is interrogated as described for single nucleotide primer extension sequencing to determine its identity. It is contemplated that degenerate probes having any form of removable 3' end block can be used in a primer extension sequencing procedure. A preferred form of removable blocking group is the cage structure, as described herein. In each case, conditions specific for removal of the particular blocking structure are used as appropriate. A preferred form of amplification and degenerate probe primer extension sequencing is Unimolecular Segment Amplification and CAGE Sequencing (USA-CAGESEQ).

D. Discrimination Between Closely Related Target Sequences

Use of a primer extension reaction to anchor a probe/primer can also be used to discriminate between two or more forms of a target sequence that differ at a particular nucleotide position. For example, different alleles of a gene differ at some nucleotide positions. By using primers where the 3' terminal nucleotide is designed to overlap a variable nucleotide position, primer extension will be dependent on hybridization between the target sequence at this position and the 3' terminal nucleotide in the primer sequence. That is, if the 3' terminal nucleotide in the primer sequence is not complementary to the nucleotide opposite it when the primer hybridizes to the target sequence, primer extension will be hampered. A particular form of a sequence (a particular allele of a gene, for example) can be detected using the disclosed method by using a probe/primer where the primer

sequence ends in a nucleotide complementary to the nucleotide present in the sequence of interest (with the rest of the primer sequence complementary to the adjacent nucleotides in the target sequence). The primer will be extended only when hybridized to a target sequence having the desired nucleotide at the critical position.

Open circle probes, gap oligonucleotides, and gap spaces can be designed to discriminate closely related target sequences, such as genetic alleles. Where closely related target sequences differ at a single nucleotide, it is preferred that open circle probes be designed with the complement of this nucleotide occurring at one end of the open circle probe, or at one of the ends of the gap oligonucleotide(s). Where gap-filling ligation is used, it is preferred that the distinguishing nucleotide appear opposite the gap space. This allows incorporation of alternative (that is, allelic) sequence into the ligated OCP without the need for alternative gap oligonucleotides. Where gap-filling ligation is used with a gap oligonucleotide(s) that partially fills the gap, it is preferred that the distinguishing nucleotide appear opposite the portion of gap space not filled by a gap oligonucleotide. Ligation of gap oligonucleotides with a mismatch at either terminus is extremely unlikely because of the combined effects of hybrid instability and enzyme discrimination. When the TS-DNA is generated, it will carry a copy of the gap oligonucleotide sequence that led to a correct ligation. Gap oligonucleotides may give even greater discrimination between related target sequences in certain circumstances, such as those involving wobble base pairing of alleles. Features of open circle probes and gap oligonucleotides that increase the target-dependency of the ligation operation are generally analogous to such features developed for use with the ligation chain reaction. These features can be incorporated into open circle probes and gap oligonucleotides for use in LM-RCA. In particular, European Patent Application EP0439182 describes several features for enhancing target-dependency in LCR that can be adapted for use in LM-RCA. The use of stop bases in the gap space, as described in European Patent Application EP0439182, is a preferred mode of enhancing the target discrimination of a gap-filling ligation operation.

A preferred form of target sequence discrimination can be accomplished by employing two types of open circle probes. In one embodiment, a single gap

oligonucleotide is used which is the same for both target sequences, that is, the gap oligonucleotide is complementary to both target sequences. In a preferred embodiment, a gap-filling ligation operation can be used. Target sequence discrimination would occur by virtue of mutually exclusive ligation events, or extension-ligation events, for which only one of the two open-circle probes is competent. Preferably, the discriminator nucleotide would be located at the penultimate nucleotide from the 3' end of each of the open circle probes. The two open circle probes would also contain two different detection tags designed to bind alternative detection probes and/or address probes. Each of the two detection probes would have a different detection label. Upon hybridization, each detection probe would produce a unique signal, for example, two alternative fluorescence colors, corresponding to the alternative target sequences.

Examples

Example 1: Target-mediated Ligation of Open Circle Probes and Rolling Circle Replication of Ligated Open Circle Probes

1. Ligation of open circle probes

Linear oligonucleotides with 5'-phosphates are efficiently ligated by ligase in the presence of a complementary target sequence. In particular, open circle probes hybridized to a target sequence and open circle probes with gap oligonucleotides hybridized to a target sequence are readily ligated. The efficiency of such ligation can be measured by LM-RCA.

The following is an example of target-dependent ligation of an open circle probe:

A DNA sample (target sample) is heat-denatured for 3 minutes at 95°C, and incubated under ligation conditions (45 minutes at 60°C) in a buffer consisting of 20 mM Tris-HCl (pH 8.2), 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM NAD, 0.05% Triton X-100, in the presence of (a) DNA ligase (AMPLIGASE®, Epicentre Technologies) at a concentration of 1 unit per 50 μl, and (b) the following 5'-phosphorylated oligonucleotides:

Open circle probe (111 nucleotides):

5'–GCCTGTCCAGGGATCTGCTCAAGACTCGTCATGTCTCAGTAGCTT CTAACGGTCACAAGCTTCTAACGGTCACAAGCTTCTAACGGTCACAT GTCTGCTGCCCTCTGTATT-3' (SEQ ID NO:1)

Gap oligonucleotide: 5'-CCTT-3'

This results in hybridization of the open circle probe and gap oligonucleotide to the target sequence, if present in the target sample, and ligation of the hybridized open circle probe and gap oligonucleotide.

2. Measuring the rate of rolling circle replication

(a) On large template: 7 kb single-stranded phage M13 circle

The rate of oligonucleotide-primed rolling circle replication on single-stranded M13 circles mediated by any DNA polymerase can be measured by using the assay described by Blanco *et al., J. Biol. Chem.* 264:8935-8940 (1989). The efficiency of primed synthesis by the ϕ29 DNA polymerase is stimulated about 3-fold in the presence of Gene-32 protein, a single-stranded DNA binding protein.

(b) On small templates: 110-nucleotide ligated open circle probes

The rate of oligonucleotide-primed rolling circle replication on single-stranded small circles of 110 bases was measured using the ϕ29 DNA polymerase generally as described in WO***LIZARDI**. After five minutes of incubation, the size of the DNA product is approximately 16 kilobases. This size corresponds to a polymerization rate of 53 nucleotides per second. The rate of synthesis with other DNA polymerases can be measured and optimized using a similar assay, as described by Fire and Xu, *Proc. Natl. Acad. Sci. USA* 92:4641-4645 (1995). It is preferred that single-stranded circles of 110 nucleotides be substituted for the 34 nucleotide circles of Fire and Xu.

The ϕ29 DNA polymerase provides a rapid rate of polymerization of the ϕ29 rolling circle reaction on 110 nucleotide circular templates. At the observed rate of 50 nucleotides per second, a 35 minute polymerization reaction will produce a DNA product of approximately 105,000 bases. This would yield an amplification of 954-fold over the original 110-base template. Fire and Xu (1995) shows that rolling circle reactions catalyzed by bacterial DNA polymerases may take place on very small circular templates of only 34 nucleotides. On the basis of

the results of Fire and Yu, rolling circle replication can be carried out using circles of less than 90 nucleotides.

Example 2: Multiplex detection of multiple target sequences using LM-RCA-CMC

This example illustrates multiplex detection using 31 different OCPs and gap oligonucleotide pairs, each designed to generate 31 different color combinations using 5 basic colors.

1. Slides containing samples are prepared as follows:

Poly-L-Lysine coated microscope slides are prepared, and DNA is spotted using an arraying machine as described above using the method described by Schena *et al.* The size of each spot of sample DNA is 2.5 mm. DNA is denatured as described above using the method described by Schena *et al.*

2. A mixture of gap oligonucleotides and open circle probes is designed and prepared, containing 31 different OCPs and 31 different gap oligonucleotides. The OCPs and gap oligonucleotides are designed as pairs with each OCP and gap probe pair containing sequences complementary to a specific target sequence of interest. The spacer regions of each of the 31 OCPs contain unique, alternative combinations of five possible detection tags, designated 1t, 2t, 3t, 4t, and 5t. The combinations are coded according to the scheme shown below. The set of pairs is designated as follows:

Gap oligo	OCP	1t	2t	3t	4t	5t
g1	ocp1	+				
g2	ocp2		+			
g3	ocp3			+		
g4	ocp4				+	
g5	ocp5					+
g6	ocp6	+	+			
..... and so on						
g25	ocp25			+	+	+
g26	ocp26	+	+	+	+	
g27	ocp27	+	+	+		+
g28	ocp28	+	+		+	+
g29	ocp29	+		+	+	+

g30	ocp30		+	+	+	+
g31	ocp31	+	+	+	+	+

3. LM-RCA is performed as follows:

The OCPs and gap oligonucleotides are hybridized and ligated to target sequences on the sample slides with 50 μl of the following mixture.

1.5 μl	10X ligation buffer (Ampligase)	
8.8 μl	BSA, 2 mg/ml stock	
15 μl	Mixture of 31 Gap oligonucleotides	[final 400 nM for each]
5 μl	Mixture of 31 OCPs	[final =100 nMolar for each]
25 μl	Ampligase (5 U/μl)	
82 μl	H_20	

The reaction is incubated for 60 minutes at 52°C.

The slides are washed twice for 5 minutes with 2X SSC with 20% formamide at 42°C, washed for two minutes with 20 mM Tris, pH 7.5, 0.075 M NaCl to remove the formamide, and washed for three minutes with 50 mM Tris, pH 7.5, 40 mM KOAc, 10 mM $MgCl_2$, 10 mM DTT, 100 μg/ml BSA.

The amplification operation is performed by placing 24 μl of the following mixture on each slide.

18.0μl	H_20 [total volume = 100 μl for 4 slides]	
20.0μl	5X φ29 buffer with BSA	BSA is 200 μg/ml
16.0μl	dNTPs (A, G, and C, each 2.5 mM)	
5.0μl	dTTP (2.5 mM)	
15.0μl	BUdR (2.5 mM)	
7.0μl	rolling circle replication primer (10 μM)	
3.0μl	Gene32 Protein (1.37 μg/μl) (final 41 μg/ml)	
16.0μl	φ29 DNA polymerase (1:6 dilution, 16 μl=768 ng)	

The reaction is incubated 15 minutes in 37°C oven.

All slides were then washed twice for four minutes with 2X SSC with 20% formamide at 25°C.

4. The 5 collapsing detection probes, each with a different label and each complementary to one of the 5 detection tags, are hybridized to the TS-DNA on the

slides in a solution of 4X SSC. The detection probes correspond to the detection tags as follows:

Detection probe	Label	Detection tag
dp1	fluorescein	1t
dp2	Cy3	2t
dp3	Cy3.5	3t
dp4	Cy5	4t
dp5	Cy7	5t

All slides were then washed twice for four minutes with 2X SSC with 20% formamide at 25°C, and then washed twice for four minutes with 2X SSC, 3% BSA, 0.1% Tween-20 at 37°C.

5. The TS-DNA generated in the amplification operation is further collapsed and detected as follows:

50 μl of a solution of AntiBUDR-Mouse.IgG (7 μg/ml) in 2X SSC, 3% BSA, 0.1% Tween-20 is placed on each slide, and the slides are incubated for 30 minutes at 37°C. Then the slides are washed three times for five minutes with 2X SSC, 3% BSA, 0.1% Tween-20 at 37°C. Next, 50 μl of a solution of Avidin DN (6 μg/ml) in 2X SSC, 3% BSA, 0.1% Tween-20 is placed on each slide, and the slides are incubated for 30 minutes at 37°C. Then the slides are washed three times for five minutes with 2X SSC, 3% BSA, 0.1% Tween-20 at 37°C, washed 5 minutes with 2X SSC, 0.01% Tween at room temperature, and then covered with 24 μl antifade. Finally, the slides are scanned in a fluorescence scanning device with appropriate filters (for example, those described by Schena *et al.*). Image analysis software is used to count and analyze the spectral signatures of the fluorescent dots.

Example 3: Detection of target sequences by target-mediated ligation of bipartite probes and ligation-mediated RCA

The following is an example of bipartite primer rolling circle amplification. This example demonstrates the use of RCA as a reporter system for quantifying hybridization/ligation events on a glass surface by single molecule analysis. The method involved target-mediated ligation of probes that hybridize at adjacent sites in a target sequence. One of the probes, referred to as a half probe, was attached to a slide and the other probe, a probe/primer having two 3' ends, was in solution.

Following ligation an amplification target circle is replicated by rolling circle amplification primed by a rolling circle replication primer sequence in the primer portion of the probe/primer. Two forms of the probe/primer were used, each complementary to a different allelic sequence in the target nucleic acid. Thus, in this example, ligation, amplification, and, ultimately, detection were determined by which allelic sequence hybridized to the half probe.

Slides were prepared containing an oligonucleotide half probe (P1), specific for a 39-base sequence adjacent to the G542X locus of the CFTR gene. The probe contained a free 5'-phosphate, and was bound covalently to the glass surface via a reactive 3'-amino group. Oligonucleotides containing a phosphate group at the 5'-end were purchased from the Yale University Critical Technologies facility. Phosphorylated P1 had the sequence: 5'-GAGAAGGTGGAATCACA CTGAGTGGAGGTCAACGAGCAATTTTTTTTTTT-(C7-NH2) (SEQ ID NO:4), where C7 is a carbon spacer. Probe P1 was immobilized over an area of 1 mm in diameter on the surface of a glass slide activated with reactive groups. The slides were coated with 4-aminobutyldimethylmethoxysilane and derivatized with 1,4-phenylene-diisothiocyanate, using the protocol of Guo *et al.*, *Nucl. Acids Res.* **22**:5456-5465 (1994), with minor modifications. Covalent coupling was obtained by reaction of a primary amine attached to the 3' end of the P1 half probe.

DNA samples were made from mixtures of mutant and wild type genomic strands of the G542X locus in different ratios to simulate the presence of rare somatic mutations. Genomic DNA preparations obtained from human cell lines either wild type or homozygous mutant at the *CFTR-G542X* locus were mixed in different pre-determined ratios (mutant/wild type equal to 1:0, 1:1, 1:25, and 1:100) and then amplified by PCR as described by Heinoven *et al.*, *Clin. Chem.* **43**:1142-1150 (1997). PCR amplicons were used as targets for allele discrimination assays on the glass slides that contained immobilized P1 molecules. The DNA was denatured at 98°C for 5 minutes immediately before use.

Two probe/primers (P2wt, P2mu) were designed, which are capable of being ligated to either the wild type or mutant locus, with precise base stacking continuity with the 5'-end of the P1 half probe. P2wt had the sequence 3'-GTTCTTGATATAACAGAAAGTTTT-(C18)-TTTTTATGATCACA GCTGAGGATAGGACATGCGA-3' (SEQ ID NO:13 and SEQ ID NO:5), where

C18 is a carbon spacer. P2mu had the sequence 3'-TTTCTTGATATAACA GAAAGTTTT-(C18)-TTTTTACGTCGTCCGTGCTAGAAGGAAACACGCA-3' (SEQ ID NO:14 and SEQ ID NO:6), where C18 is a carbon spacer. An allele-specific base was located at a 3'-hydroxyl terminus in these probes for optimal discrimination in the ligation step. The opposite end of these probe/primers contained a coded primer sequence (corresponding to one of two alternative primers) with a free 3'-OH terminus, obtained by reversal of backbone polarity during chemical synthesis.

The ligation of Pl with the allele-specific probe/primers P2wt or P2mu was accomplished as shown in Figure 2, and generated a surface-bound oligonucleotide with a free 3'-terminus, competent for coded priming of an RCA reaction. This hybridization/ligation was carried out in 7.5µl of 20 mM Tris-Cl, 50 mM KC1, 10 mM $MgCl_2$, 10 mM DTT, 1 mM NAD, 200 µg/ml BSA and 0.5 unit/µl of Ampligase (Epicentre). Target concentration was in the range of 0.1 nM to 0.2 nM. Probe/primers P2wt and P2mu were 1 µM each. All enzymatic reactions took place by confining the reaction volume over the DNA dot with a small silicone rubber O-ring sealed with rubber cement. The slide was covered with a second slide, forming a sandwich filled with wet filter paper perforated at the positions of the O-rings. This sandwich was placed on an aluminum block in a pre-heated moisture chamber. Ligation took place at 62°C for 2 hours. Washes were performed at 70°C, as follows: twice for 3 minutes in 75% formamide + 2X SSPE (1X SSPE: 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 0.1% SDS, 1 min in 0.05% Triton X-100, 2 min with 6X SSPE, 0.05% Triton X-100, 1 min in 0.01% Triton X-100 and finally a brief rinse in water.

Two different amplification target circles were used for RCA, one (Cwt) designed to be complementary to the primer sequence of P2wt, the other (Cmu) to the primer sequence of P2mu. Cwt had the sequence CGCATGTCCTATCCT CAGCTGTGATCATCAGAACTCACCTGTTAGACGCCACCAGCTCCAACT GTGAAGATCGCTTAT (SEQ ID NO:7). Cmu had the sequence GCGTGTTT CCTTCTAGCACGGACGACGTATATGATGGTACCGCAGCCAGCATCACC AGACTGAGTATCTCCTATCACT (SEQ ID NO:8). Each of these two amplification target circles can be replicated in a rolling circle amplification process mediated by its cognate primer -- in the event, and only in the event, that

the complementary primer became covalently bound to the surface in the ligation reaction. That is, the P2wt probe/primer primes RCA only of one of the amplification target circles (Cwt), and the P2mu probe/primer primes RCA only of the other amplification target circle (Cmu). In the absence of ligation, no priming can occur since unligated probe/primers are removed before RCA.

Hybridization of the amplification target circles (Cwt and Cmu) to ligated probes was performed using a concentration of 170 nM for each circle in 6 μl of Buffer 1 (67 mM Tris-Cl, pH 8.0, 16.7 mM $MgCl_2$, 84 mM NaCl, 8 mM DTT, 0.0167% Triton X-100). After 30 min at 45°C, the slide was placed on ice, 2 μl of Buffer II (containing 20 μM E. Coli SSB, 25 mM DTT, and 1.5 mM each of dATP, dCTP, dGTP and dTTP) was added, and incubation proceeded for 5 min at 37°C. RCA reactions were carried out by adding 2 μl of 5 μM Sequenase Version 2.0 DNA polymerase was added, followed by incubation at 37°C for 20 minutes. Stringent washes at 70°C were as follows: twice for 3 minutes in 75% formamide, 2X SSC, 0.1% SDS (1X SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4), 1 min in 0.2X SSC + 0.05% Triton X-100, 2 min in 4X SSC, 0.05% Triton X-100.

The DNA generated by RCA, tandem sequence DNA (TS-DNA), was labeled with fluorescent DNP-oligonucleotide tags (that is, collapsing detection probes) that hybridize at multiple sites in the tandem DNA sequence. The "decorated" TS-DNA, labeled by specific encoding tags, was then collapsed (that is, condensed into a small object) by cross-linking with a multivalent anti-DNP IgM. The wild-type specific primer (P2wt) generated RCA products to which fluorescein-labeled DNP-collapsing detection probes were hybridized, while the mutant RCA products (generated by the P2mu primer) were hybridized to Cy3-labeled DNP-collapsing detection probes. The collapsing detection probe Fl-det1c-DNP had the sequence FITC-TCAGAACTCACCTGTTAG-3'-DNP (SEQ ID NO:9). The collapsing detection probe Fl-det1d-DNP had the sequence FITC-ACTGTGAAGATCGCTTAT-3'-DNP (SEQ ID NO:10). The collapsing detection probe Cy3-det2b-DNP had the sequence Cy3-TATATGATG GTACCGCAG-3'-DNP (SEQ ID NO:11). The collapsing detection probe Cy3-det2c-DNP had the sequence Cy3-TGAGTATCTCCTATCACT-3'-DNP (SEQ ID NO:12).

To collapse and detect the amplified DNA, each dot on the slide was covered with 7.5 µl of collapsing detection probes (0.5 µM each of Fl-det1c-DNP, F1-det1d-DNP, Cy3-det2b-DNP,Cy3-det2c-DNP in 2X SSC + 0.05% Triton X-100 + 0.5 mg/ml degraded herring sperm DNA), and incubated at 37°C for 20 minutes. The slide was washed 4 times for 5 minutes with 2X SSC + 0.01% Tween 20, and 4 times for 5 minutes with 4X SSC + 0.05% Triton X-100, rinsed once with 2X SSC and drained. 7.5 µl of condensation solution (33 µM mouse anti-DNP IgM in 2X SSC, 0.1% Tween 20, 0.5% BSA, 1 mg/ml degraded herring sperm DNA) was added, and the slide was incubated at 37°C for 15 min. And washed twice for 5 min in 2X SSC, 0.1% Tween 20 at room temperature, drained, and covered with Pro-long antifade (Molecular Probes Inc.) under a cover slip.

Fluorescent imaging was performed using a Zeiss epifluorescence microscope equipped with a Photometrics cooled CCD camera. For an oligonucleotide spot of 1 mm in diameter, the maximum number of non-overlapping green or red signals can be calculated to be about 80,000 using these conditions. The images show many hundreds of fluorescent dots, with a diameter of 0.2 to 0.5 microns. The ratio of fluorescein-labeled to Cy3-labeled dots shown in Table 1 corresponds closely to the known ratio of mutant to wild type strands, down to a value of 1/100.

Table 1

Wt/Mu ratio	Green counts	Red counts	Total counts	FITC/Cy3 ratio
1 to 0	4613	9	4622	513
1 to 1	2315	2093	4408	1.1
25 to 1	2758	107	2865	26
100 to 1	4799	46	4845	104

A few red signals were generated by pure wild type DNA (ratio=1/512), and these were interpreted as resulting from mismatch ligation events, which re expected to occur with a frequency of 1/500 to 1/1500 when using wild type *T. Thermophilus* DNA ligase (Luo *et al.*, *Nucl. Acids Res.* **24**:3071-3078 (1996)).

CLAIMS

We claim:

1. A method of amplifying nucleic acid sequences, the method comprising,

(a) mixing a half probe with a target sample comprising a target sequence, to produce a probe-target mixture, and incubating the probe-target mixture under conditions that promote hybridization between the half probe and the target sequence in the probe-target mixture,

(b) mixing a probe/primer with the probe-target mixture, and incubating the probe-target mixture under conditions that promote hybridization between the probe/primer and the target sequence,

(c) mixing ligase with the probe-target mixture, to produce a ligation mixture, and incubating the ligation mixture under conditions that promote ligation of the half probe and the probe/primer to form a rolling circle replication primer,

(d) mixing an amplification target circle with the rolling circle replication primer, to produce a primer-ATC mixture, and incubating the primer-ATC mixture under conditions that promote hybridization between the amplification target circle and the rolling circle replication primer in the primer-ATC mixture, and

(e) mixing DNA polymerase with the primer-ATC mixture, to produce a polymerase-ATC mixture, and incubating the polymerase-ATC mixture under conditions that promote replication of the amplification target circle,

wherein replication of the amplification target circle results in the formation of tandem sequence DNA.

2. The method of claim 1 wherein the half probe is immobilized on a solid-state support.

3. The method of claim 1 wherein the probe/primer has two free 3' ends and no free 5' end.

4. The method of claim 1 wherein the primer portion and the target probe portion of the probe/primer are coupled 5' end to 5' end.

5. The method of claim 1 wherein the nucleotides of the primer portion of the probe/primer are all in the 3' to 5' orientation and the nucleotides of the target probe portion of the probe/primer are all in the 5' to 3' orientation.

6. The method of claim 1 wherein the half probe and the probe/primer hybridize to adjacent regions in the target sequence.

7. The method of claim 1 wherein the half probe and the probe/primer hybridize to different regions in the target sequence such that there is a gap space between the half probe and the probe/primer when they are hybridized to the target sequence,

wherein, prior to ligation, the gap space is filled by hybridization of one or more gap oligonucleotides, by gap-filling synthesis, or by a combination of one or more gap oligonucleotides and gap-filling synthesis.

8. The method of claim 1 further comprising detecting the tandem sequence DNA, wherein detection of the tandem sequence DNA indicates the presence of the target sequence in the target sample.

9. The method of claim 1 wherein the half probe comprises a target probe portion,

wherein the probe/primer comprises a primer portion and a target probe portion,

wherein the target sequence comprises a 5' region and a 3' region, wherein the 5' region and the 3' region are adjacent in the target sequence, and

wherein the target probe portion of the half probe and the target probe portion of the probe/primer are complimentary to the 5' region and the 3' region, respectively, of the target sequence.

10. The method of claim 1 wherein a plurality of different half probes are mixed with the target sample,

wherein a plurality of different probe/primers are mixed with the probe-target mixture,

wherein the target sample contains a plurality of target sequences,

wherein each half probe and each probe/primer hybridize to adjacent regions in at least one of the target sequences,

wherein a plurality of the half probes and a plurality of the probe/primers are ligated to form a plurality of different rolling circle replication primers,

wherein the hybridized amplification target circles are replicated to form a plurality of tandem sequence DNA molecules.

11. The method of claim 10 further comprising detecting the tandem sequence DNA molecules, wherein detection of the tandem sequence DNA molecules indicates the presence of the target sequences in the target sample.

12. The method of claim 10 further comprising detecting the tandem sequence DNA molecules, wherein detection of the tandem sequence DNA molecules indicates the presence of the corresponding target sequences in the target sample.

13. The method of claim 10 wherein the plurality of probe/primers comprise four sets of probe/primers,

wherein each probe/primer comprises a primer portion and a target probe portion,

wherein each probe/primer has the structure

3'-Pr-L-n_jN_kX-3'

wherein Pr is the primer portion,

n_jN_kX is the target probe portion,

L is either a covalent bond or a linker between the primer portion and the target probe portion,

n represents a universal nucleoside,

j is 2-20,

N represents one of the nucleotides A, C, G or T,

k is 3, 4, 5, 6, 7, or 8,

X represents one of the nucleotides A, C, G or T,

wherein each N is randomized within each set of probe/primers,

wherein X represents the same nucleotide in all of the probe/primers in a set of probe/primers,

wherein X represents a different nucleotide in each of the four sets.

14. The method of claim 13 wherein the nucleotides of the primer portion of the probe/primer are all in the 3' to 5' orientation and the nucleotides of the target probe portion of the probe/primer are all in the 5' to 3' orientation.

15. The method of claim 1 wherein a plurality of different half probes are mixed with the target sample, wherein the half probe comprises a target probe portion,

wherein a plurality of different probe/primers are mixed with the probe-target mixture, wherein the probe/primer comprises a primer portion and a target probe portion,

wherein the target sample contains a plurality of target sequences, wherein each target sequence comprises a 5' region and a 3' region, wherein the 5' region and the 3' region are adjacent in each target sequence,

wherein the target probe portion of each half probe and the target probe portion of each probe/primer are complimentary to the 5' region and the 3' region, respectively, of at least one of the target sequences,

wherein a plurality of the half probes and a plurality of the probe/primers are ligated to form a plurality of different rolling circle replication primers,

wherein the hybridized amplification target circles are replicated to form a plurality of tandem sequence DNA molecules.

16. The method of claim 15 further comprising detecting the tandem sequence DNA molecules, wherein detection of the tandem sequence DNA molecules indicates the presence of the corresponding target sequences in the target sample.

17. A method of amplifying nucleic acid sequences, the method comprising,

(a) mixing a probe/primer with a target sample comprising a target sequence, to produce a probe-target mixture, and incubating the probe-target mixture under conditions that promote hybridization between the probe/primer and the target sequence.

wherein the target sequence is immobilized on a solid-state support,

wherein the probe/primer comprises a primer portion and a target probe portion,

wherein the probe/primer has two free 3' ends and no free 5' end,

(b) mixing a polymerase with the probe-target mixture, to produce an extension mixture, and incubating the extension mixture under conditions that promote extension of the probe/primer from the target probe portion of the probe/primer,

(c) mixing an amplification target circle with the probe/primer, to produce a primer-ATC mixture, and incubating the primer-ATC mixture under conditions that

promote hybridization between the amplification target circle and the rolling circle replication primer in the primer-ATC mixture, and

(d) mixing DNA polymerase with the primer-ATC mixture, to produce a polymerase-ATC mixture, and incubating the polymerase-ATC mixture under conditions that promote replication of the amplification target circle,

wherein replication of the amplification target circle results in the formation of tandem sequence DNA,

wherein the target sequence DNA is immobilized on the solid-state support via hybridization between the extended target probe portion of the probe/primer and the target sequence.

18. The method of claim 17 wherein the target sample is immobilized through cytological or histological preparation.

19. The method of claim 17 wherein the 3' terminal nucleotide in the target probe portion of the probe/primer corresponds to a nucleotide position in the target sequence that varies in different forms of the target sequence.

20. A method of detecting a nucleic acid of interest, the method comprising

(a) bringing into contact a target sample containing a target sequence and a probe/primer and incubating under conditions that promote hybridization between the probe/primer and the target sequence,

(b) mixing an amplification target circle with the hybridized probe/primer and incubating under conditions that promote hybridization between the amplification target circle and the probe/primer, and

(c) mixing DNA polymerase with the hybridized amplification target circle and incubating the polymerase-ATC mixture under conditions that promote replication of the amplification target circle,

wherein replication of the amplification target circle results in the formation of tandem sequence DNA,

(d) detecting the tandem sequence DNA molecules, wherein detection of the tandem sequence DNA molecules indicates the presence of the target sequences in the target sample.

21. A kit comprising

(a) a probe/primer, wherein the probe/primer comprises a primer portion and a target probe portion, wherein the nucleotides of the primer portion of the probe/primer are all in the 3' to 5' orientation and the nucleotides of the target probe portion of the probe/primer are all in the 5' to 3' orientation,

(b) a half probe, wherein the half probe comprises a target probe portion,

wherein the target probe portion of the half probe and the target probe portion of the probe/primer are complimentary to a target sequence, wherein the target sequence comprises a 5' region and a 3' region, and wherein the target probe portion of the half probe and the target probe portion of the probe/primer are complimentary to the 5' region and the 3' region, respectively, of the target sequence.

22. The kit of claim 21 comprising a plurality of probe/primers and a plurality of half probes.

23. The kit of claim 21 wherein the 5' region and the 3' region are adjacent in the target sequence.

24. The kit of claim 21 wherein the 5' region and the 3' region are separated such that there is a gap space between the half probe and the probe/primer when they are hybridized to the target sequence.

25. A kit comprising four sets of probe/primers.

wherein each probe/primer comprises a primer portion and a target probe portion,

wherein each probe/primer has the structure

3'-Pr-L-n_jN_kX-3'

wherein Pr is the primer portion,

n_jN_kX is the target probe portion,

L is either a covalent bond or a linker between the primer portion and the target probe portion,

n represents a universal nucleoside,

j is 2-20,

N represents one of the nucleotides A, C, G or T,

k is 3, 4, 5, 6, 7, or 8,

X represents one of the nucleotides A, C, G or T,

wherein each N is randomized within each set of probe/primers,

wherein X represents the same nucleotide in all of the probe/primers in a set of probe/primers,

wherein X represents a different nucleotide in each of the four sets.

26. The kit of claim 25 wherein the nucleotides of the primer portion of the probe/primer are all in the 3' to 5' orientation and the nucleotides of the target probe portion of the probe/primer are all in the 5' to 3' orientation.

27. The kit of claim 25 further comprising a plurality of amplification target circles,

wherein each amplification target circle comprises a primer complement portion,

wherein the primer complement portion of each amplification target circle is complementary to the primer portion of one or more of the probe/primers.

28. The kit of claim 27 further comprising a plurality of collapsing detection probes each comprising a sequence matching a sequence in one or more of the amplification target circles.

29. The kit of claim 28 wherein all of the probe/primers in each set of probe/primers has the same primer portion, wherein the primer portion is different for each set of probe/primers.

wherein there are four amplification target circles, each with a different primer complement portion complementary to one of the primer portions,

wherein there are four collapsing detection probes, each comprising a different sequence matching a sequence in one of the amplification target circles.

30. A method of amplifying nucleic acid sequences, the method comprising,

(a) mixing one or more different open circle probes with a target sample comprising one or more target sequences, to produce an OCP-target sample mixture,

wherein the target sequences each comprise a 5' region and a 3' region,

wherein the open circle probes each comprise a single-stranded, linear DNA molecule comprising, from 5' end to 3' end, a 5' phosphate group, a right target probe portion, a spacer portion, a left target probe portion, and a 3' hydroxyl group, wherein the spacer portion comprises a primer complement portion, and wherein the left target probe portion and the right target probe

portion of the same open circle probe are each complementary to the 3' region and the 5' region, respectively, of the same target sequence,

wherein at least one of the target sequences further comprises a central region located between the 5' region and the 3' region,

wherein neither the left target probe portion of the open circle probe nor the right target probe portion of any of the open circle probes is complementary to the central region of the target sequences, and

incubating the OCP-target sample mixture under conditions that promote hybridization between the open circle probes and the target sequences in the OCP-target sample mixture,

(b) mixing ligase and DNA polymerase with the OCP-target sample mixture, to produce a ligation mixture, and incubating the ligation mixture under conditions that promote ligation of the open circle probes to form amplification target circles, wherein during incubation the DNA polymerase fills in the central region of the target sequences,

(c) mixing one or more rolling circle replication primers with the ligation mixture, to produce a primer-ATC mixture, and incubating the primer-ATC mixture under conditions that promote hybridization between the amplification target circles and the rolling circle replication primers in the primer-ATC mixture,

wherein the one or more rolling circle replication primers are immobilized on a solid-state support, and

(d) mixing DNA polymerase with the primer-ATC mixture, to produce a polymerase-ATC mixture, and incubating the polymerase-ATC mixture under conditions that promote replication of the amplification target circles,

wherein replication of the amplification target circle results in the formation of tandem sequence DNA.

31. The method of claim 30 wherein sequences in the one or more rolling circle replication primers match the central region of one or more of the target sequences.

ADDRESS PROBE HYBRIDIZING TO TS-DNA PORTION BRIDGING GAP OLIGONUCLEOTIDE AND TARGET PROBE ENDS

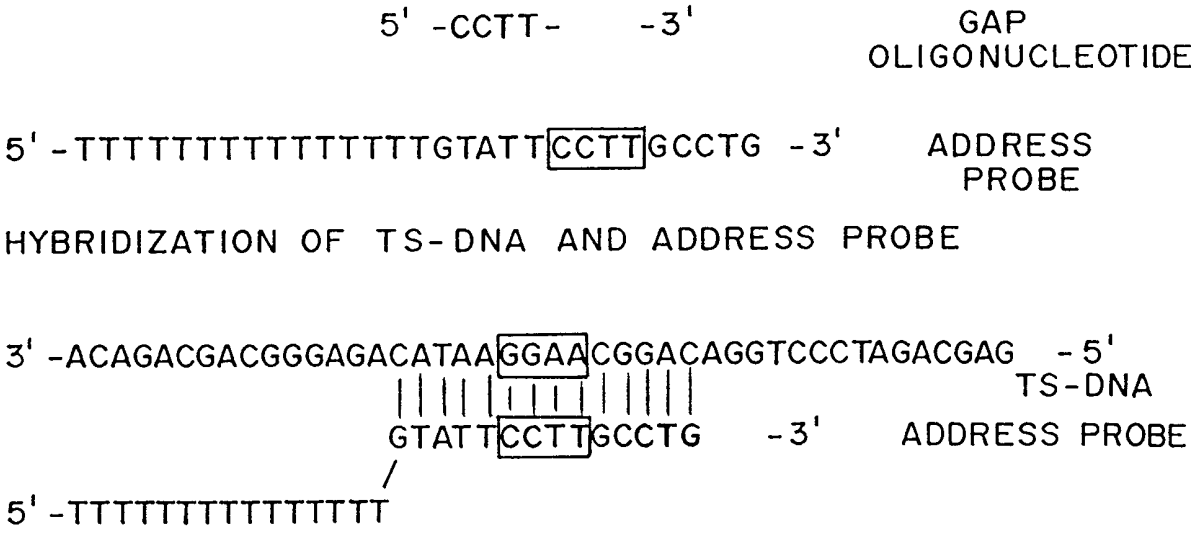

FIG. 1

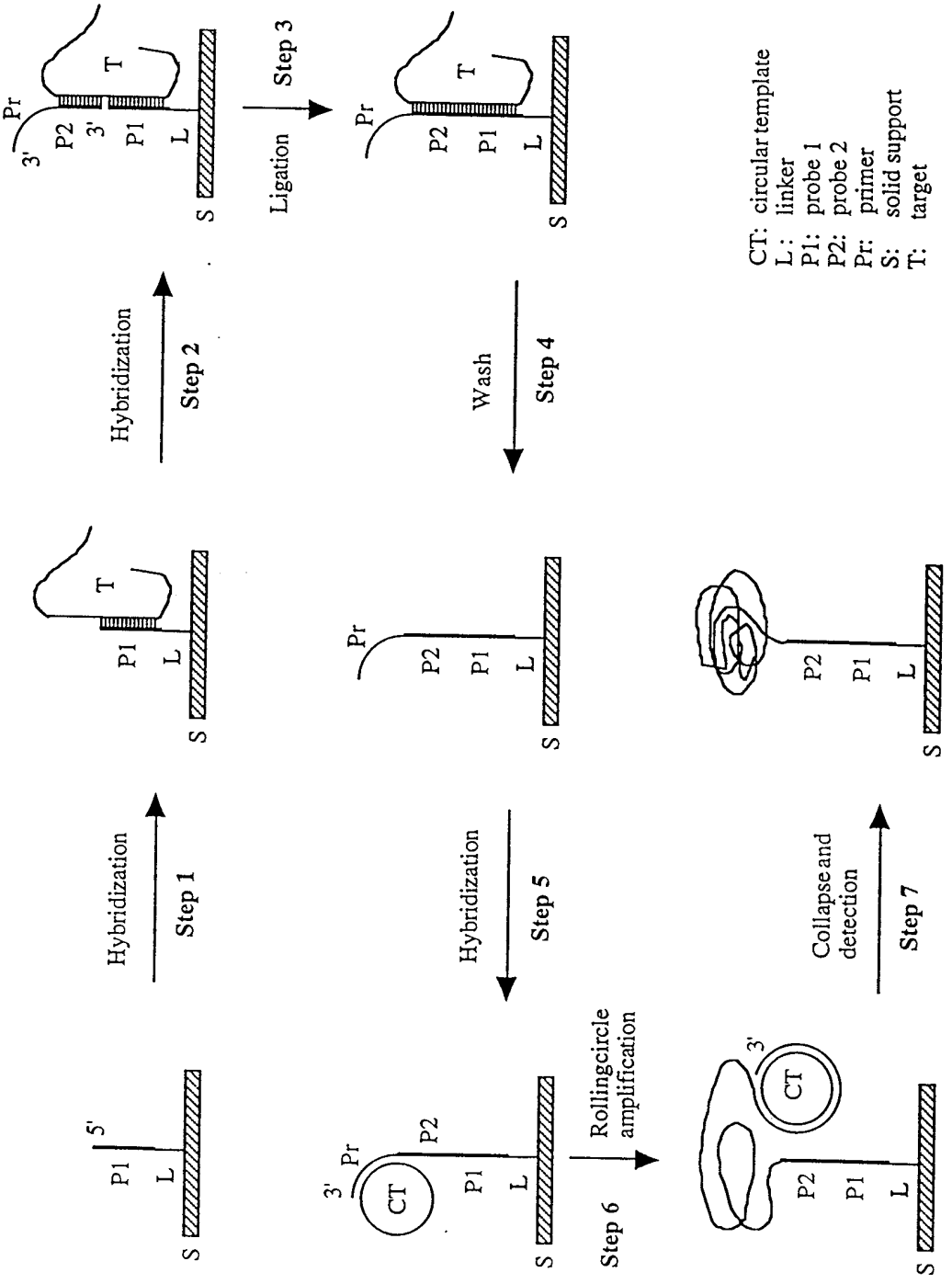
Step 1
Hybridization
Step 2
Hybridization
Step 3
Ligation
Step 4
Wash
Step 5
Hybridization
Step 6
Rollingcircle amplification
Step 7
Collapse and detection
CT: circular template
L.: linker
P1: probe 1
P2: probe 2
Pr: primer
S: solid support
T: target

FIG. 2

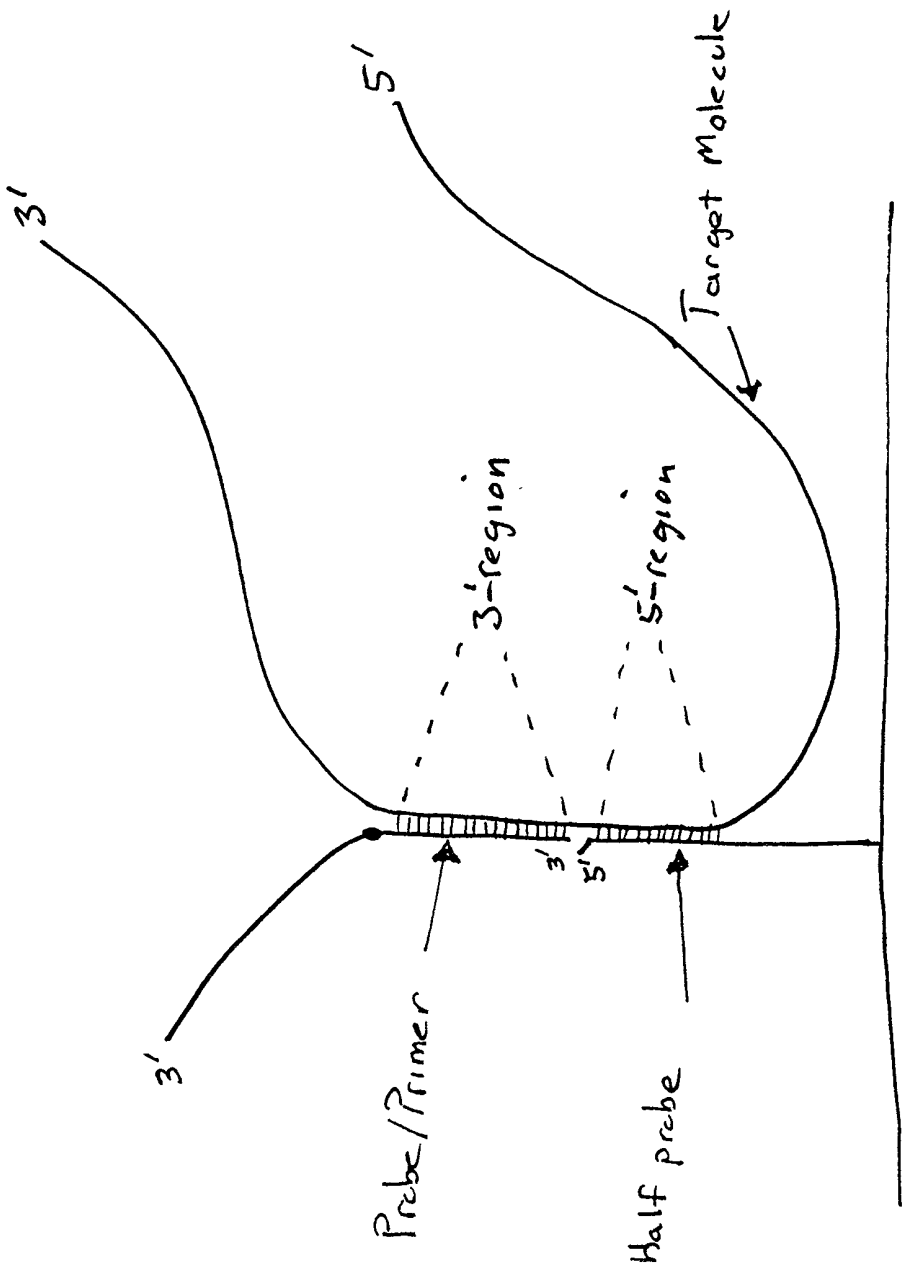
5'
Target Molecule
3'
3'-region
5'-region
3'
5'
3'
Probe/Primer
Half probe

FIG. 3

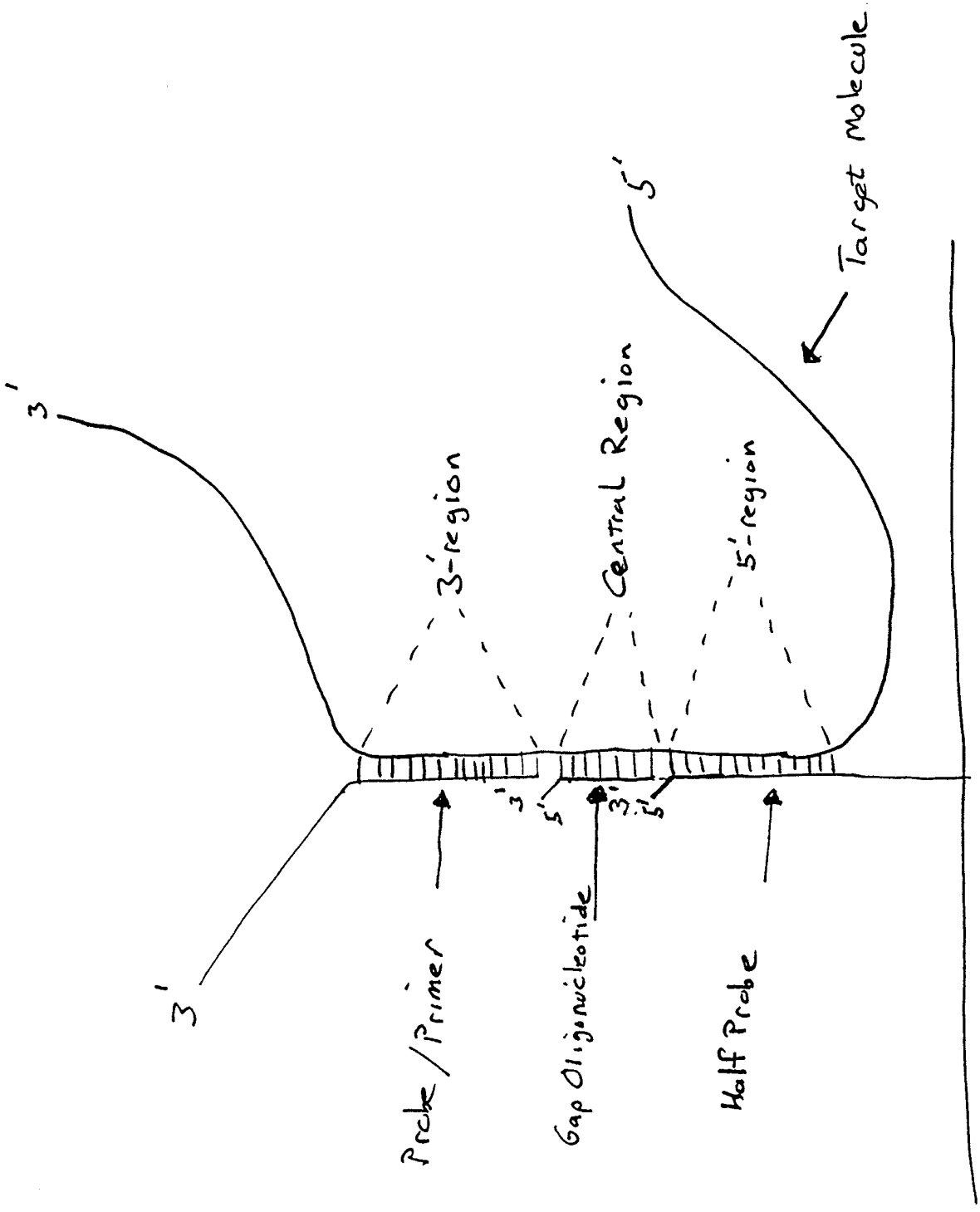
Target Molecule
5'
3'
3'-region
Central Region
5'-region
3'
5'
3'
5'
3'
Probe / Primer
Gap Oligonucleotide
Half Probe

Fig.4

SEQUENCE LISTING

<110> Yale University

<120> Single Molecule Analysis Using Target-Mediated Ligation
of Bipartite Primers

<130> YU 123 PCT

<140> PCT/US99
<141> 1999-06-20

<150> 60/093,479
<151> 1998-06-20

<160> 14

<170> PatentIn Ver. 2.0

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<213> Artificial Sequence

<220>
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probe

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ttctaacggt cacaagcttc taacggtcac atgtctgctg ccctctgtat t 111

<210> 2
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<220>
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sequence DNA (TS-DNA)

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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: address probe

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<210> 4
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<213> Artificial Sequence

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P1

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<220>
<223> Description of Artificial Sequence: Probe/primer
P2wt

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<210> 6
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<213> Artificial Sequence

<220>
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P2mu

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target circle Cwt

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actgtgaaga tcgcttat 78

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<223> Description of Artificial Sequence: amplification
target circle Cmu

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tgagtatctc ctatcact 78

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<220>
<223> Description of Artificial Sequence: collapsing
detection probe F1-det1c-DNP

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tcagaactca cctgttag 18

<210> 10
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: collapsing
detection probe F1-det1d-DNP

<400> 10
actgtgaaga tcgcttat 18

<210> 11
<211> 18
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: collapsing detection probe Cy3-det2b-DNP

<400> 11
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<210> 12
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: collapsing detection probe Cy3-det2c-DNP

<400> 12
tgagtatctc ctatcact 18

<210> 13
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Probe/primer P2wt

<400> 13
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<210> 14
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Probe/primer P2mu

<400> 14
ttttgaaaga caatatagtt cttt 24

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 99/16373

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 97 19193 A (UNIV YALE) 29 May 1997 (1997-05-29) cited in the application the whole document	1-31
Y	WO 94 24312 A (BECKMAN INSTRUMENTS INC) 27 October 1994 (1994-10-27) page 10, paragraph 2 -page 13, paragraph 2; claims 1-17; example 1	1-31
Y	WO 94 16108 A (PUBLIC HEALTH RESEARCH INST OF) 21 July 1994 (1994-07-21) figures 7,9; example 1	1-31
Y	EP 0 379 369 A (SYNTEX INC) 25 July 1990 (1990-07-25) claims 8-18	1-31

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 1999	02/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl. Fax: (+31–70) 340–3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 99/16373

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication.where appropriate. of the relevant passages	Relevant to claim No.
A	WO 98 04746 A (SINAI SCHOOL MEDICINE) 5 February 1998 (1998-02-05) the whole document	1-31
A	US 5 648 245 A (FIRE ANDREW ET AL) 15 July 1997 (1997-07-15)	
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INTERNATIONAL SEARCH REPORT

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Int[illegible]onal Application No
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