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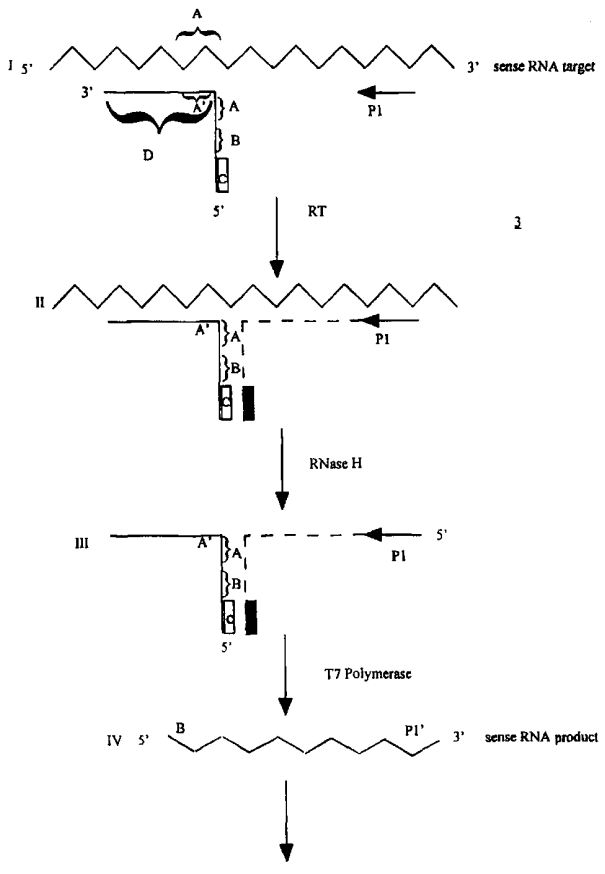
(57) Abstract

An isothermal, transcription–based nucleic acid amplification method based on the formation of a target–dependent nucleic acid species by template switching. This product species can be amplified further to produce multiple copies of both double stranded DNA products and single stranded RNA product. The single stranded RNA amplification products are the same sense as the target sequence. Also provided is a branch migration inhibition based procedure for scanning nucleic acid sequences using the strand switch isothermal amplification. A template switch oligonucleotide used in the amplification includes a 3' region capable of hybridizing to a target sequence and a 5' region which does not hybridize to the target. The 5' region includes a promoter sequence of DNA dependent RNA polymerase. A first primer and a second primer may be used during amplification and detection. The first primer is capable of hybridizing to the target and the second primer is not target dependent. For detection, either the first primer or the second primer has a label.



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HOMOGENEOUS ISOTHERMAL AMPLIFICATION AND DETECTION OF NUCLEIC ACIDS USING A TEMPLATE SWITCH OLIGONUCLEOTIDE

FIELD OF THE INVENTION

The present invention relates to the detection of differences in nucleic acids using a method for isothermal amplification of polynucleotide sequences. The amplification method uses template switch oligonucleotide which subsequently allows for the detection of the presence of a difference between a target polynucleotide sequence and a reference polynucleotide sequence.

BACKGROUND OF THE INVENTION

Significant morbidity and mortality are associated with infectious diseases. More rapid and accurate diagnostic methods are required for better monitoring and treatment of disease. Molecular methods using DNA probes, nucleic acid hybridizations and *in vitro* amplification techniques are promising methods offering advantages to conventional methods used for patient diagnoses.

Nucleic acid hybridization has been employed for investigating the identity and establishing the presence of nucleic acids. Hybridization is based on complementary base pairing. When complementary single stranded nucleic acids are incubated together, the complementary base sequences pair to form double stranded hybrid molecules. The ability of single stranded deoxyribonucleic acid (ssDNA) or ribonucleic acid (RNA) to form a hydrogen bonded structure with a complementary nucleic acid sequence has been employed as an analytical tool in molecular biology research. The availability of radioactive nucleoside triphosphates of high specific activity and the ^{32}P labeling of DNA with T4 polynucleotide kinase has made it possible to identify, isolate, and characterize various nucleic acid sequences of biological interest.

Nucleic acid hybridization has great potential in diagnosing disease states associated with unique nucleic acid sequences. These unique nucleic acid sequences may result from genetic or environmental change in DNA by insertions, deletions, point mutations, or by acquiring foreign DNA or RNA by means of infection by bacteria, molds, fungi, and viruses. Nucleic acid hybridization has, until now, been employed primarily in academic and industrial molecular biology laboratories. The application of nucleic acid hybridization as a diagnostic tool in clinical medicine is limited because of the frequently very low concentrations of disease related DNA or RNA present in a

patient's body fluid and the unavailability of a sufficiently sensitive method of nucleic acid hybridization analysis.

One method for detecting specific nucleic acid sequences generally involves immobilization of the target nucleic acid on a solid support such as nitrocellulose paper, cellulose paper, diazotized paper, or a nylon membrane. After the target nucleic acid is fixed on the support, the support is contacted with a suitably labeled probe nucleic acid for about two to forty-eight hours. After the above time period, the solid support is washed several times at a controlled temperature to remove unhybridized probe. The support is then dried and the hybridized material is detected by autoradiography or by spectrometric methods. When very low concentrations must be detected, this method is slow and labor intensive, and nonisotopic labels that are less readily detected than radiolabels are frequently not suitable.

Various *in vitro* nucleic acid amplification procedures have been described in recent years. Some of the amplification methods are dependent on thermal cycling (PCR, LCR, and SPA) and others are isothermal (NASBA, TMA, Q beta replicase and SDA). Among the advantages of isothermal amplification are speed, simplification of instrumentation, and ease of integration with fully automated analyzers. NASBA, TMA, and other variations of transcription-based amplification procedures afford amplification of single-stranded nucleic acid target molecules to produce multiple copies of a single-stranded RNA product. However, these methods utilize two primers which are complementary to opposite sense, single-stranded target or product. The use of the two primers of opposite polarity impairs the ability to selectively amplify a specific strand of a nucleic acid target.

Another drawback of the current transcription-based, isothermal, amplification methods is the lack of control of initiation of the amplification process. Amplification of an RNA target is initiated by hybridization of a primer which is 5'-tailed by a sequence representing one strand of a DNA-dependent RNA polymerase promoter. The hybridization complex serves as a priming complex for a reverse transcriptase to produce an RNA-DNA heteroduplex. The RNA strand of the heteroduplex is degraded by RNase H to produce a single-stranded DNA product. A second reverse primer hybridizes to the newly formed DNA molecule at a site downstream to the first primer sequence and is extended to form a double-stranded DNA molecule. This process produces a double-stranded promoter site for the DNA-dependent RNA polymerase

which in turn produces single-stranded RNA products, which are anti-sense to the initial target. The rate of initiation of this process is not well-controlled and could pose a problem when attempting to quantify a target nucleic acid when present in very low concentration.

Also, the exact mechanism for the amplification of either double-stranded DNA target molecules or a single-stranded DNA target using the current isothermal transcription-based amplification methods is not yet definitely determined. This impairs the ability to specifically amplify RNA target in the presence of excess DNA. The specific amplification of an RNA target is required for the detection and quantification of mRNA, as is required for analysis of gene expression, as well as the detection of free RNA viruses in the presence of host cells which are likely to contain integrated viral genes.

Regardless of the amplification used, the amplified product must be detected. One method for detecting nucleic acids is to employ nucleic acid probes. A method utilizing such probes is described in U.S. Patent No. 4,868,104. A nucleic acid probe may be, or may be capable of being, labeled with a reporter group or may be, or may be capable of becoming, bound to a support. Detection of signal depends upon the nature of the label or reporter group. If the label or reporter group is an enzyme, additional members of the signal producing system include enzyme substrates and so forth.

Also, PCT application WO 97/23646, incorporated fully herein by reference, describes a method for detection of sequence alteration based on inhibition of DNA branch migration. This method is based on inhibition of spontaneous strand exchange by branch migration in four-stranded DNA cruciform structures when a test sequence is altered relative to a reference sequence. The substrates are produced by PCR amplification of test and reference DNA sequences using specifically modified primers. Any sequence alterations, such as base substitutions, deletions, and insertions are equally detected, and the method is useful for the detection of sequence alterations in heterozygote and homozygote genotypes. In addition to its potential usefulness for the diagnosis of genetic disease, the method is also useful for the determination of sequence identity required for various applications.

The branch migration inhibition method for detection of sequence alteration requires the formation of amplification products which are capable, upon denaturation

and re-association, of forming partial duplexes which in turn anneal to form four-stranded cruciform structures. When the four-stranded cruciform structures are formed in a mixture of test and reference amplification products, strand exchange by spontaneous branch migration proceeds if the test and reference amplification products are identical. When the test sequence is different from the reference, branch migration is inhibited, resulting in the formation of stable, detectable four stranded cruciform structures.

The recent advances in gene analysis methodology created the need for rapid, high-throughput methods for identification and quantification of specific nucleic acid sequences as well as detection of sequence alterations and the sequence determination where alteration is detected. It is desirable to have a sensitive, simple method for amplifying and detecting nucleic acids, preferably in an isothermal and homogeneous format. The method should minimize the number and complexity of steps and reagents. Also, such method should have ability to selectively amplify a specific strand of a nucleic acid target as well as control the rate of initiation of this process. Further, the ability to specifically amplify RNA target in the presence of excess DNA is highly desirable.

Description of the Related Art

The polymerase chain reaction (PCR) was described by Saiki, R. et al, Enzymatic Amplification of β-Globin Genomic Sequences and Restriciton Site Analysis for Diagnosis of Sickle Cell Anemia, *Science*, 230:1350-1354 (1985); Mullis, K. et al, Specific Synthesis of DNA *In Vitro* Via A Polymerase-Catalyzed Chain Reaction, *Methods in Enzymology*, 155:335-350 (1987) and U.S. Patent Nos. 4,683,195, 4,683,202, 04,800,159, 4,465,188 and 5,008,182.

Ligase Chain Reaction (LCR) is described in European Patent Application No. 0320308B1, as well as Wu D. et al, The Ligation Amplification Reaction (LAR) -- Amplification of Specific DNA Sequences Using Sequential Rounds of Template-Dependent Ligation, *Genomics*, 4:560-569 (1989) and Barany, F., Genetic disease detection and DNA amplification using cloned thermostable ligase, *Proc. Natl. Acad. Sci.*, 88:189-193 (1991).

Nucleic acid amplification using single polynucleotide primer (ASPP) is described in U.S. Patent No. 5,595,891.

A method for producing a single stranded polynucleotide having two different defined sequences and kits for use in ASPP is described in U.S. Patent No. 5,683,879 and U.S. Patent No. 5,679,512.

Homogeneous amplification and detection of nucleic acids in a sealed tube is described in WO 97/23547.

The mechanism for template switch amplification is described in Patel, R. et al, Formation of chimeric DNA primer extension products by template switching onto an annealed downstream oligonucleotide, *Proc. Natl. Acad. Sci. USA*, 93:2969-2974 (1996).

A method of introducing defined sequences at the 3' end of a polynucleotide using a template switch oligonucleotide is described in U.S. Patent No. 5,679,512.

Amplification of specific RNA using RNA directed polymerase such as Q beta replicase, is described in U.S. Patent No. 4,786,600.

Transcription based amplification of nucleic acid sequences is described in U.S. Patent No. 5,766,849 (TMA) and U.S. Patent No. 5,654,142 (NASBA).

The 3SR transcription based amplification (equivalent to NASBA) is described in Guatelli, J. et al, Isothermal, *in vitro* amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication, *Proc. Natl. Acad. Sci. USA*, 87:1874-1878 (1990).

Early description of amplification schemes using a primer containing a promoter sequence is provided in PCT WO 89/01050.

Another isothermal amplification method, Strand Displacement Amplification, is described by Fraiser, et al, in U.S. Patent No. 5,648,211. The method is also described in Walker, G. *et al.*, Isothermal *in vitro* amplification of DNA by a restriction enzyme/DNA polymerase system, *Proc. Natl. Acad. Sci. USA*, 89:392-396 (1992).

Chemiluminscece based homogenous detection of single standed products, Hybridization Protection Assay or HPA, is described in PCT US88/02746.

Chemiluminescence based detection of SDA products is described in Walker *et al.*, *Mol. Methods Virus Detect.*, Publisher Academic, San Diego CA, Conference, 329-349 (1995).

Another technique for homogenous detection of single stranded amplification products using fluorescence correlation spectroscopy is described in Oehlenschlager, F. et al, Detection of HIV-1 RNA by nucleic acid sequence-based amplification

combined with fluorescence correlation spectroscopy, *Proc. Natl. Acad. Sci. USA*, 93:12811-12816 (1996).

Electrochemiluminescence-based detection is described by DiCesare, J. et al, A High-Sensitivity Electrochemiluminescence-Based Detection System for Automated PCR Product Quantitation, *BioTechniques*, 15(1):152-157 (1993).

Rapid, non-separation electrochemiluminescent DNA hybridization assays for PCR products using 3'-labeled oligonucleotide probes is described by Gudibande, *et al.*, *Molecular and Cellular Probes*, 6:495-503. (1992). A related disclosure is found in international patent application WO 95/08644 A1).

Marmaro, *et al.*,(Meeting of the American Association of Clinical Chemists, San Diego, California, November 1994, Poster No. 54) discusses the design and use of fluorogenic probes in TaqMan, a homogeneous PCR assay.

A PCR-based assay that utilizes the inherent 5' nuclease of rTth DNA polymerase for the quantitative detection of HCV RNA is disclosed by Tsang, *et al.*, (94th General Meeting of the American Society for Microbiology, Las Vegas NE 5/94, Poster No. C376).

Kemp, *et al.*, *Gene*, 94:223-228 (1990), discloses simplified colorimetric analysis of polymerase chain reactions and detection of HIV sequences in AIDS patients.

German patent application DE 4234086-A1 (92.02.05) (Henco, *et al.*) discusses the determination of nucleic acid sequences amplified *in vitro* in enclosed reaction zone where probe(s) capable of interacting with target sequence is present during or after amplification and spectroscopically measurable parameters of probe undergo change thereby generating signal.

U.S. Patent No. 5,232,829 (Longiaru, *et al.*) discloses detection of chlamydia trachomatis by polymerase chain reaction using biotin labeled DNA primers and capture probes. A similar disclosure is made by Loeffelholz, *et al. Journal of Clinical Microbiology,* 30(11):2847-2851 (1992).

Padlock probes, circularizing oligonucleotides for localized DNA detection, are described by Nilsson, *et al. Science*, 265:2085-2088 (1994).

Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase is described by Saiki, *et al.*, *Science,* 239:487 (1988).

U.S. Patent No. 5,508,178 describes nucleic acid amplification using a single polynucleotide primer (ASPP). U.S. Patent No. 5,595,891 discloses methods for

producing a polynucleotide for use in single primer amplification. U.S. Patent No. 5,439,793 describes a method for producing a molecule containing an intramolecular base-pair structure. A method for producing a polynucleotide for use in single primer amplification is described in U.S. Patent No. 5,612,199. A method for introducing defined sequences at the 3'-end of a polynucleotide is described in U.S. Patent No. 5,679,512.

Formation of a single base mismatch that impedes spontaneous DNA branch migration is described by Panyutin, *et al.*, (1993) *J. Mol. Biol.*, 230:413-424.

The kinetics of spontaneous DNA branch migration is discussed by Panyutin, *et al.*, (1994) *Proc. Natl. Acad. Sci. USA*, 91:2021-2025.

European Patent Application No. 0 450 370 A1 (Wetmur, *et al.*) discloses branch migration of nucleotides.

A displacement polynucleotide assay method and polynucleotide complex reagent therefor is discussed in U.S. Patent No. 4, 766,062 (Diamond, *et al.*).

A strand displacement assay and complex useful therefor is discussed in PCT application WO 94/06937 (Eadie, *et al*).

PCT application WO/86/06412 (Fritsch, *et al.*) discusses process and nucleic acid construct for producing reagent complexes useful in determining target nucleotide sequences.

SUMMARY OF THE INVENTION

The present invention provides for a method of producing multiple copies of a nucleic acid sequence involving the step of combining in a target polynucleotide, a first oligonucleotide primer, a template switch oligonucleotide, and reagents sufficient for conducting and amplification of the polynucleotide sequence. The combination is subjected to conditions for amplifying the polynucleotide sequence. The template switch oligonucleotide includes a 3' region capable of hybridizing to the target and a 5' region which does not hybridize to the target. The 5' region includes (1) a propromoter sequence of a DNA dependant RNA polymerase, (2) a sequence substantially homologous to the target sequence located 3' of the propromoter sequence, and (3) a region unrelated to the target sequence located between the propomoter sequence and the sequence homologous to the target.

The invention further provides for the addition of a second primer unrelated to the target sequence. The second primer includes a sequence substantially

homologous to the 5' sequence of the template switch oligonucleotide which is unrelated to the target. When the template is DNA, the invention provides for a denaturation step.

In another aspect of the invention, a first primer and a template switch oligonucleotide are hybridized to the same strand target. The first primer is extended along the target and then along the template switch oligonucleotide to form a complex comprising a first extension product and the promoter for a DNA dependent RNA polymerase. The first extension product is transcribed to produce multiple copies of a first transcription product which includes a sequence substantially homologous to the target.

The first primer may hybridize to the transcription product and be extended to form an RNA/DNA heteroduplex comprising a second extension product. After degradation of the transcription product, the second primer is hybridized to the second extension product and is extended to form a third extension product. The second extension product is also extended to produce a fully double stranded DNA product with the third extension product. The double stranded DNA product is transcribed to produce multiple copies of the transcription product which includes a sequence substantially homologous to the target.

Another aspect of the invention involves the detection of the presence of a difference between a target nucleic acid sequence and a reference sequence. The target and the reference nucleic acid sequences are amplified using a template switch oligonucleotide, a first primer, a second primer and a third primer. The first and second primers have common 3' sequences which are complementary to the target sequence and the reference sequence, and 5' tails which are not complementary to the target, the reference, or each other. In this primer scheme, either (1) the first primer has a first label and the second primer has a second label, or (2) the third primer is a mixture of the third primer with the first label and the third primer with the second label. After amplification, a complex is formed including the reference sequence and the target sequence in double stranded form. The complex has at least one pair of non-complementary strands and each of the non-complementary strands has a label. The complex is subjected to strand exchange conditions wherein if no difference exists between the reference sequence and the target sequence, strand exchange continues until complete. If a difference is present, strand exchange in the complex ceases. The

association of the first label and the second label is detected. The association of the labels being related to the presence of the difference.

Labels useful for the present invention include oligonucleotides, enzymes, dyes, fluorescent molecules, co-enzymes, enzyme substrates, radioactive groups, small organic molecules, polynucleotide sequences and solid surfaces. The detection method includes the detection of a difference between a target nucleic acid and a reference nucleic acid when the difference is a mutation. In one aspect of the invention, the complex includes a Holliday junction.

A further aspect of the present invention is a kit comprising, along with standard reagents, the oligonucleotides of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1a, 1b and 2 are schematic diagrams depicting the method of producing multiple copies of a target RNA or single stranded DNA using a template switch oligonucleotide according to the present invention.

FIG. 3 is a schematic diagram generally representing an example of the branch migration inhibition method of detection of a mutation in a nucleic acid sequence.

FIGS. 4a and 4b a schematic diagrams depicting the amplification method of the present invention using labeled primers and primers with oligonucleotide tails according to the present invention.

FIG. 5 is a schematic diagram depicting the double stranded DNA substrates (partial duplexes) for forming the signal generating cruciform structures of the present invention.

FIG. 6 is a schematic diagram depicting generation of the signal generating cruciform structures of the present invention.

FIG. 7 is a schematic diagram depicting the detection of the amplification products using two labeled probes.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes an isothermal, transcription-based nucleic acid amplification method which is based on the formation of unique target-dependent nucleic acid species by template switching. This product species can be amplified further to produce both double-stranded DNA products and multiple copies of a single-

stranded RNA product. The single-stranded RNA amplification products are of the same sense as a target sequence.

The new amplification method of this invention provides a well-defined mechanism for DNA target sequence amplification and further requires denaturation of a double-stranded DNA target prior to the amplification of a specific sequence. Thus, the new method provides means for specific amplification of an RNA sequence in the presence of double-stranded DNA target. The new method leads to a more controlled formation of the substrate for the DNA-dependent RNA polymerase, and thus to a better control of the kinetics of amplification.

The invention further provides a process for performing analysis of sequence alteration, or genotyping, using detection by the branch migration inhibition method as described in WO 97/23646.

Before proceeding further with a description of the specific embodiments of the present invention, a number of terms will be defined.

Nucleic acid -- a compound or composition that is a polymeric nucleotide or polynucleotide. The nucleic acids include both nucleic acids and fragments thereof from any source, in purified or unpurified form including DNA (dsDNA and ssDNA) and RNA, including t-RNA, m-RNA, r-RNA, mitochondrial DNA and RNA, chloroplast DNA and RNA, DNA-RNA hybrids, or mixtures thereof, genes, chromosomes, plasmids, the genomes of biological material such as microorganisms, e.g., bacteria, yeasts, viruses, viroids, molds, fungi, plants, animals, humans, cDNA, and the like. The nucleic acid can be only a minor fraction of a complex mixture such as a biological sample. The nucleic acid can be obtained from a biological sample by procedures well known in the art. Also included are genes, such as hemoglobin gene, cystic fibrosis gene, oncogenes, and the like. Where the nucleic acid is RNA, it is first converted to cDNA by means of a primer and reverse transcriptase. The nucleotide polymerase used in the present invention for carrying out amplification and chain extension can have reverse transcriptase activity. Sequences of interest may be embedded in sequences of any length of the chromosome, cDNA, plasmid, etc.

Sample -- the material suspected of containing the nucleic acid. Such samples include biological fluids such as blood, serum, plasma, sputum, lymphatic fluid, semen, vaginal mucus, feces, urine, spinal fluid, and the like; biological tissue such as hair and skin; and so forth. Other samples include cell cultures and the like, plants, food,

forensic samples such as paper, fabrics and scrapings, water, sewage, medicinals, etc. When necessary, the sample may be pretreated with reagents to liquefy the sample and release the nucleic acids from binding substances. Such pretreatments are well known in the art.

Amplification of nucleic acids -- any method that results in the formation of one or more copies of a nucleic acid. Numerous methods are known including the polymerase chain reaction (PCR), ligase chain reaction (LCR), amplification using Q beta replicase, nucleic acid sequence based amplification (NASBA), single primer amplification (ASPP) and others. References to these and other amplification methods are provided in the "Description of the Related Art" section, *supra.*

Chain extension of nucleic acids -- extension of the 3'-end of a polynucleotide in which additional nucleotides or bases are appended. Chain extension relevant to the present invention is template dependent, that is, the appended nucleotides are determined by the sequence of a template nucleic acid to which the extending chain is hybridized. The chain extension product sequence that is produced is complementary to the template sequence. Usually, chain extension is catalyzed by a nucleotide polymerase.

Target nucleic acid sequence (test nucleic acid sequence) -- a sequence of nucleotides to be studied either for the presence of a difference from a related sequence or for the determination of its presence or absence. The target nucleic acid sequence may be double stranded or single stranded and from a natural or synthetic source. The target sequence usually exists within a portion or all of a nucleic acid, the identity of which is known to an extent sufficient to allow preparation of various primers necessary for introducing one or more priming sites flanking the target sequence or conducting an amplification of the target sequence or a chain extension of the products of such amplification in accordance with the present invention. Accordingly, other than for the sites to which the primers bind, the identity of the target nucleic acid sequence may or may not be known. In general, in PCR, primers hybridize to, and are extended along (chain extended), at least the target sequence, and, thus, the target sequence acts as a template. The target sequence usually contains from about 30 to 20,000 or more nucleotides, more frequently, 100 to 10,000 nucleotides, preferably, 50 to 1,000 nucleotides. The target nucleic acid sequence is generally a fraction of a larger molecule or it may be substantially the entire molecule.

The minimum number of nucleotides in the target sequence is selected to assure that a determination of a difference between two related nucleic acid sequences in accordance with the present invention can be achieved.

Reference nucleic acid sequence -- a nucleic acid sequence that is related to the target nucleic acid in that the two sequences are identical except for the presence of a difference, such as a mutation. Where a mutation is to be detected, the reference nucleic acid sequence usually contains the normal or "wild type" sequence. In certain situations the reference nucleic acid sequence may be part of the sample as, for example, in samples from tumors, the identification of partially mutated microorganisms, or identification of heterozygous carriers of a mutation. Consequently, both the reference and the target nucleic acid sequences are subjected to similar or the same amplification conditions. As with the target nucleic acid sequence, the identity of the reference nucleic acid sequence need be known only to an extent sufficient to allow preparation of various primers necessary for introducing one or more priming sites flanking the reference sequence or conducting an amplification of the target sequence or a chain extension of the products of such amplification in accordance with the present invention. Accordingly, other than for the sites to which the primers bind, the identity of the reference nucleic acid sequence may or may not be known. The reference nucleic acid sequence may be a reagent employed in the methods in accordance with the present invention. Depending on the method of preparation of this reagent it may or may not be necessary to know the identity of the reference nucleic acid. The reference nucleic acid reagent may be obtained form a natural source or prepared by known methods such as those described below in the definition of oligonucleotides.

Holliday junction -- the branch point in a four way junction in a complex of two nucleic acid sequences and their complementary sequences. The junction is capable of undergoing branch migration resulting in dissociation into two double stranded sequences where sequence identity and complementarity extend to the ends of the strands.

Quadromolecular Complex -- a complex of four nucleic acid strands containing a Holliday junction, which is inhibited from dissociation into two double stranded sequences because of a difference in the sequences and their complements.

Related nucleic acid sequences -- two nucleic acid sequences are related when they contain at least 15 nucleotides at each end that are identical but have different lengths or have intervening sequences that differ by at least one nucleotide. Frequently, related nucleic acid sequences differ from each other by a single nucleotide. Such difference is referred to herein as the "difference between two related nucleic acid sequences." A difference can be produced by the substitution, deletion or insertion of any single nucleotide or a series of nucleotides within a sequence.

Mutation -- a change in the sequence of nucleotides of a normally conserved nucleic acid sequence resulting in the formation of a mutant as differentiated from the normal (unaltered) or wild type sequence. Mutations can generally be divided into two general classes, namely, base-pair substitutions and frameshift mutations. The latter entail the insertion or deletion of one to several nucleotide pairs. A difference of one nucleotide can be significant as to phenotypic normality or abnormality as in the case of, for example, sickle cell anemia.

Duplex -- a double stranded nucleic acid sequence wherein all, or substantially all, of the nucleotides therein are complementary.

Oligonucleotide -- a single stranded polynucleotide, usually a synthetic polynucleotide. The oligonucleotide(s) are usually comprised of a sequence of 10 to 100 nucleotides, preferably, 20 to 80 nucleotides in length.

Various techniques can be employed for preparing an oligonucleotide utilized in the present invention. Such oligonucleotide can be obtained by biological synthesis or by chemical synthesis. For short sequences (up to about 100 nucleotides) chemical synthesis will frequently be more economical as compared to the biological synthesis. In addition to economy, chemical synthesis provides a convenient way of incorporating low molecular weight compounds and/or modified bases during the synthesis step. Furthermore, chemical synthesis is very flexible in the choice of length and region of the target polynucleotide binding sequence. The oligonucleotide can be synthesized by standard methods such as those used in commercial automated nucleic acid synthesizers. Chemical synthesis of DNA on a suitably modified glass or resin can result in DNA covalently attached to the surface. This may offer advantages in washing and sample handling. For longer sequences standard replication methods

employed in molecular biology can be used such as the use of M13 for single stranded DNA as described by Messing J., *Methods Enzymol*, 101:20-78 (1983).

Other methods of oligonucleotide synthesis include phosphotriester and phosphodiester methods, Narang, *et al,. Meth. Enzymol,* 68:90 (1979) and synthesis on a support, Beaucage, *et al., Tetrahedron Letters,* 22:1859-1862 (1981), as well as phosphoramidate technique, Caruthers, M. H., *et al.*, *Methods in Enzymology*, 154:287-314 (1988), and others described in *Synthesis and Applications of DNA and RNA*, S.A. Narang, editor, Academic Press, New York, 1987, and the references contained therein.

Oligonucleotide primer(s) -- an oligonucleotide that is usually employed in a chain extension on a polynucleotide template such as in, for example, an amplification of a nucleic acid. The oligonucleotide primer is usually a synthetic oligonucleotide that is single stranded, containing a hybridizable sequence at its 3'-end that is capable of hybridizing with a defined sequence of the target or reference polynucleotide. Normally, the hybridizable sequence of the oligonucleotide primer has at least 90%, preferably 95%, most preferably 100%, complementarity to a defined sequence or primer binding site. The number of nucleotides in the hybridizable sequence of an oligonucleotide primer should be such that stringency conditions used to hybridize the oligonucleotide primer will prevent excessive random non-specific hybridization. Usually, the number of nucleotides in the hybridizable sequence of the oligonucleotide primer will be at least ten nucleotides, preferably at least 15 nucleotides and, preferably 20 to 50, nucleotides. In addition, the primer may have a sequence at its 5'-end that does not hybridize to the target or reference polynucleotides that can have 1 to 60 nucleotides, preferably, 8 to 30 polynucleotides.

Nucleoside triphosphates -- nucleosides having a 5'-triphosphate substituent. The nucleosides are pentose sugar derivatives of nitrogenous bases of either purine or pyrimidine derivation, covalently bonded to the 1'-carbon of the pentose sugar, which is usually a deoxyribose or a ribose. The purine bases comprise adenine(A), guanine (G), inosine (I), and derivatives and analogs thereof. The pyrimidine bases comprise cytosine (C), thymine (T), uracil (U), and derivatives and analogs thereof. Nucleoside triphosphates include deoxyribonucleoside triphosphates such as the four common triphosphates dATP, dCTP, dGTP and dTTP and ribonucleoside triphosphates such as the four common triphosphates rATP, rCTP, rGTP and rUTP.

The term "nucleoside triphosphates" also includes derivatives and analogs thereof, which are exemplified by those derivatives that are recognized and polymerized in a similar manner to the underivatized nucleoside triphosphates. Examples of such derivatives or analogs, by way of illustration and not limitation, are those which are biotinylated, amine modified, alkylated, and the like and also include phosphorothioate, phosphite, ring atom modified derivatives, and the like.

Nucleotide -- a base-sugar-phosphate combination that is the monomeric unit of nucleic acid polymers, i.e., DNA and RNA.

Nucleoside -- is a base-sugar combination or a nucleotide lacking a phosphate moiety.

Nucleotide polymerase -- a catalyst, usually an enzyme, for forming an extension of a polynucleotide along a DNA or RNA template where the extension is complementary thereto. The nucleotide polymerase is a template dependent polynucleotide polymerase and utilizes nucleoside triphosphates as building blocks for extending the 3'-end of a polynucleotide to provide a sequence complementary with the polynucleotide template. Usually, the catalysts are enzymes, such as DNA polymerases, for example, prokaryotic DNA polymerase (I, II, or III), T4 DNA polymerase, T7 DNA polymerase, Klenow fragment, and reverse transcriptase, and may be thermally stable DNA polymerases such as Vent® DNA polymerase, VentR® DNA polymerase, *Pfu*® DNA polymerase, *Taq*® DNA polymerase, and the like, derived from any source such as cells, bacteria, such as *E. coli*, plants, animals, virus, thermophilic bacteria, and so forth.

Wholly or partially sequentially -- when the sample and various agents utilized in the present invention are combined other than concomitantly (simultaneously), one or more may be combined with one or more of the remaining agents to form a subcombination. Subcombination and remaining agents can then be combined and can be subjected to the present method.

Hybridization (hybridizing) and binding -- in the context of nucleotide sequences these terms are used interchangeably herein. The ability of two nucleotide sequences to hybridize with each other is based on the degree of complementarity of the two nucleotide sequences, which in turn is based on the fraction of matched complementary nucleotide pairs. The more nucleotides in a given sequence that are complementary to another sequence, the more stringent the conditions can be for

hybridization and the more specific will be the binding of the two sequences. Increased stringency is achieved by elevating the temperature, increasing the ratio of cosolvents, lowering the salt concentration, and the like.

Complementary -- Two sequences are complementary when the sequence of one can bind to the sequence of the other in an anti-parallel sense wherein the 3' end of each sequence binds to the 5'-end of the other sequence and each A, T(U), G, and C of one sequence is then aligned with a T(U), A, C, and G, respectively, of the other sequence. "Complementary" does not require that sequences have 100% base pairing. Sequences capable of hybrizing to each other, although having base pair mismatches, are complementary for the purposes of this invention.

Copy -- means a sequence that is a direct identical copy of a single stranded polynucleotide sequence as differentiated from a sequence that is complementary to the sequence of such single stranded polynucleotide.

Conditions for extending a primer -- includes a nucleotide polymerase, nucleoside triphosphates or analogs thereof capable of acting as substrates for the polymerase and other materials and conditions required for enzyme activity such as a divalent metal ion (usually magnesium), pH, ionic strength, organic solvent (such as formamide), and the like.

Member of a specific binding pair ("sbp member") -- one of two different molecules, having an area on the surface or in a cavity which specifically binds to and is thereby defined as complementary with a particular spatial and polar organization of the other molecule. The members of the specific binding pair are referred to as ligand and receptor (antiligand). These may be members of an immunological pair such as antigen-antibody, or may be operator-repressor, nuclease-nucleotide, biotin-avidin, hormone-hormone receptor, IgG-protein A, DNA-DNA, DNA-RNA, and the like.

Ligand -- any compound for which a receptor naturally exists or can be prepared.

Receptor ("antiligand") -- any compound or composition capable of recognizing a particular spatial and polar organization of a molecule, e.g., epitopic or determinant site. Illustrative receptors include naturally occurring and synthetic receptors, e.g., thyroxine binding globulin, antibodies, Fab fragments thereof, enzymes, lectins, nucleic acids, repressors, oligonucleotides, protein A, complement component C1q, or DNA binding proteins and the like.

Small organic molecule -- a compound of molecular weight less than about 1500, preferably 100 to 1000, more preferably 300 to 600 such as biotin, digoxin, fluorescein, rhodamine and other dyes, tetracycline and other protein binding molecules, and haptens, etc. The small organic molecule can provide a means for attachment of a nucleotide sequence to a label or to a support.

Support or surface -- a porous or non-porous water insoluble material. The support can be hydrophilic or capable of being rendered hydrophilic and includes inorganic powders such as silica, magnesium sulfate, and alumina; natural polymeric materials, particularly cellulosic materials and materials derived from cellulose, such as fiber containing papers, e.g., filter paper, chromatographic paper, etc.; synthetic or modified naturally occurring polymers, such as nitrocellulose, cellulose acetate, poly (vinyl chloride), polyacrylamide, cross linked dextran, agarose, polyacrylate, polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), nylon, poly(vinyl butyrate), etc.; either used by themselves or in conjunction with other materials; glass available as Bioglass, ceramics, metals, and the like. Natural or synthetic assemblies such as liposomes, phospholipid vesicles, and cells can also be employed.

Binding of sbp members to a support or surface may be accomplished by well-known techniques, commonly available in the literature. See, for example, *Immobilized Enzymes,* Ichiro Chibata, Halsted Press, New York (1978) and Cuatrecasas, *J.Biol.Chem.*, 245:3059 (1970). The surface can have any one of a number of shapes, such as strip, rod, particle, including bead, and the like.

Label -- a member of a signal producing system. Labels include reporter molecules that can be detected directly by virtue of generating a signal, and specific binding pair members that may be detected indirectly by subsequent binding to a cognate that contains a reporter molecule such as oligonucleotide sequences that can serve to bind a complementary sequence or a specific DNA binding protein; organic molecules such as biotin or digoxigenin that can bind respectively to streptavidin and anti-digoxin antibodies, respectively; polypeptides; polysaccharides; and the like. In general, any reporter molecule that is detectable can be used. The reporter molecule can be isotopic or nonisotopic, usually non-isotopic, and can be a catalyst, such as an enzyme, dye, fluorescent molecule, chemiluminescer, coenzyme, enzyme substrate, radioactive group, a particle such as latex or carbon particle, metal sol, crystallite,

liposome, cell, etc., which may or may not be further labeled with a dye, catalyst or other detectable group, and the like. The reporter group can be a fluorescent group such as fluorescein, a chemiluminescent group such as luminol, a terbium chelator such as N-(hydroxyethyl) ethylenediaminetriacetic acid that is capable of detection by delayed fluorescence, and the like.

The label is a member of a signal producing system and can generate a detectable signal either alone or together with other members of the signal producing system. As mentioned above, a reporter molecule can serve as a label and can be bound directly to a nucleotide sequence. Alternatively, the reporter molecule can bind to a nucleotide sequence by being bound to an sbp member complementary to an sbp member that comprises a label bound to a nucleotide sequence. Examples of particular labels or reporter molecules and their detection can be found in U.S. Patent 5,595,891, the relevant disclosure of which is incorporated herein by reference.

Signal Producing System -- the signal producing system may have one or more components, at least one component being the label. The signal producing system generates a signal that relates to the presence of the analyte or the presence of a difference between the target polynucleotide sequence and the reference polynucleotide sequence. The signal producing system includes all of the reagents required to produce a measurable signal. When a reporter molecule is not conjugated to a nucleotide sequence, the reporter molecule is normally bound to a sbp member complementary to a sbp member that is bound to or is part of a nucleotide sequence. Other components of the signal producing system can include substrates, enhancers, activators, chemiluminescent compounds, cofactors, inhibitors, scavengers, metal ions, specific binding substances required for binding of signal generating substances, coenzymes, substances that react with enzymic products, enzymes and catalysts, and the like. The signal producing system provides a signal detectable by external means, such as by use of electromagnetic radiation, electrochemical detection, desirably by spectrophotometric detection.

Ancillary Materials -- Various ancillary materials will frequently be employed in the methods and assays carried out in accordance with the present invention. For example, buffers will normally be present in the assay medium, as well as stabilizers for the assay medium and the assay components. Frequently, in addition to these additives, proteins may be included, such as albumins, organic solvents such as

formamide, quaternary ammonium salts, polycations such as dextran sulfate, surfactants, particularly non-ionic surfactants, binding enhancers, e.g., polyalkylene glycols, or the like.

Template Switch Isothermal Amplification

In general, template switching refers to switching of polymerase catalyzed primer extension from the original target template to a segment of an oligonucleotide, a template switch oligonucleotide, which is annealed to the target strand downstream from the primer annealing site. The novel amplification method of the present invention utilizes two oligonucleotide (DNA) primers, P1 and P2, and one template switch oligonucleotide (DNA). Primer P1 and the template switch oligonucleotide (TSO) are able to hybridize to the same single-strand RNA or DNA target. While an RNA single-stranded target is readily amplifiable by the new procedure, amplification of a double-stranded DNA target requires a denaturation step prior to amplification to yield a single-stranded target for the subsequent hybridization of P1 and the TSO.

Referring now to FIG. 1a, Primer P1, is composed of a sequence complementary to the target. The TSO is composed of two sections: the 3' portion, D, which is complementary to the target, and a 5'-tail portion. This 5'-portion of the TSO is composed of three sections: A, B, and C. Sequence A, is complementary to sequence A' of the target complementary section of the TSO and is, in turn, the same as an A sequence of the target. The A' portion of the TSO is at the 5'-end of the target complementary portion, D, of the TSO.

The design parameters for this section of the TSO, A' and A are described in detail in Patel R, et al, Formation of chimeric DNA primer extension by template switching onto an annealed downstream oligonucleotide, *PNAS*, 93:2969-74 (1996) and U.S. Patent No. 5,679,512. Briefly, the efficiency of template switching is influenced by the sequence element of the TSO and by reaction conditions. The switch of primer extension from the original template to the unhybridized portion of the TSO results from initial primer extension into the double stranded portion at the site of hybridization of the TSO to the target strand, with concomitant displacement of a portion of the TSO. As primer extension proceeds through the double stranded region, a competition is set up between the 3' end of the extending strand and the segment of the displaced TSO for hybridization to the target template, which reaches an equilibrium

states. The two partners of this equilibrium are (1) the fully hybridized primer extension product and the partially hybridized TSO (with portion of the A' region of the target complementary portion displaced from hybridization to the target strand by the primer extension product), and (2) fully hybridized TSO and partially hybridized primer extension product, where the 3' most portion of the extending strand is not hybridized to the target strand (displace by the TSO). The 3' region of the primer extension product in the last case can hybridize to the A region of the TSO.

The hybridization of the primer extension product to the TSO segment results in formation of a thermodynamically stable tri molecular complex and leads to disruption of the equilibrium described above in favor of the second partner. Primer extension then proceeds along the TSO strand and template switching is accomplished. Primer extension into the downstream double stranded portion usually extends to 1 to 10 bases, dependent on the sequence content and the temperature of the reaction. Thus extension into a GC rich segment is more limited than extension into an AT rich segment. In order to achieve efficient extension into the double stranded segment and subsequent template switching, the A portion should comprise of about 10 nucleotides and should be moderately AT rich.

Sequence B of the TSO as described herein, which is immediately 5' to A, is not related to the target sequence. Sequence C, which is immediately 5' to sequence B, is the same as a single stranded (pro-promoter) sequence of the promoter of the DNA-dependent RNA polymerase. Referring now to FIG. 1b, Primer P2 is composed of sequences B and C and is identical to the B and C sequence at the 5'-end of the TSO. Sequence B may be any sequence which is not related to the target and represents the most optimal sequence for the DNA-dependent RNA polymerase used. In cases when it is desired to limit the length of the B sequence, it is possible to include in the 3'-end of P2 a few of the 5'-end residues of section A of the TSO. It is desirable to limit the number of the A nucleotides in the P2 sequence so as to make it substantially non-complementary to the target sequence. This restriction will ensure that P2 is unable to hybridize to the initial target molecule and be subsequently extended by the reverse transcriptase enzyme.

Amplification of an RNA Target

Either two or three enzymes are used in the amplification reaction: (1) reverse transcriptase, capable of synthesizing the complement of either an RNA or a DNA single-stranded target molecule by extending a primer hybridized to the target molecule, (2) an RNase H which degrades an RNA strand of an RNA/DNA heteroduplex, however, the RNase H activity may reside in the reverse transcriptase enzyme or could be a separate entity, and (3) an RNA polymerase which requires a double-stranded DNA promoter sequence for production of an RNA product complementary to a DNA template molecule.

A sample suspected of containing the specific RNA target is mixed with the appropriate buffer, primers P1 and P2, the TSO, and NTPs (dNTPs and rNTPs), as required for transcription-based amplification. The mixture is heated to 65°C for a short period, to allow denaturation of secondary structures in the RNA target. The mixture is then incubated at 41°C (or the temperature suitable for activity of the enzymes used for target amplification). Dependent on the T_m of the target complementary sequences of the TSO and P1, these oligonucleotides will hybridize to the target at either 65°C or 41°C, to form complex I (FIG. 1a). P1 hybridizes to the target at a position which is 3' to the TSO hybridization sequence. Following a short incubation at 41°C, the amplification enzymes are added.

Referring to FIG. 1a, the reverse transcriptase extends the P1 primer along the target molecule, up to the site of TSO hybridization. A template switch will occur at this site, as previously described (Patel R. et al., 1996, *supra*). Primer extension then follows along the TSO single-stranded template to produce the complement of the 5'-portion of TSO (which is not complementary to the target). This process results in the production of three-stranded structure, complex II, which includes a double-stranded DNA portion forming the promoter of the DNA-dependent RNA polymerase. The RNase H enzyme activity then degrades the RNA target in the portions of the RNA/DNA hybrid to form complex III.

The DNA-dependent RNA polymerase then binds to the double-stranded promoter sequence to transcribe the newly formed template-switch DNA product, producing a plurality of an RNA transcription product, IV, which is the same sense as the initial RNA target molecule.

The next sequence of reactions, shown in FIG. 1b, results in the formation of a double-stranded DNA product, which is a substrate for the DNA-dependent RNA polymerase, to produce additional RNA products similar to the products produced in the initial sequence of reactions. Thus, a cycle for exponential amplification of the initial target nucleic acid is established.

Referring now to FIG 1b, Primer P1 hybridizes to the P1' sequence at the 3'-end of the RNA product molecule, V. RT then extends the primer to replicate the RNA product, resulting in formation of an RNA/DNA heteroduplex VI. RNase H then degrades the RNA molecule of the heteroduplex, resulting in formation of a single-stranded DNA product VII. Primer P2 hybridizes to the B' sequence at the 3'-end of the DNA product to form complex VIII. RT then extends P2 to replicate the DNA product. In addition, RT also extends the 3' end of the single-stranded DNA product to form a fully double-stranded promoter.

The double-stranded DNA product, IX, is a substrate for the DNA-dependent RNA polymerase, to produce multiple copies of the single-stranded RNA product IV. This last product, IV, is a substrate for formation of the double-stranded DNA product, leading to exponential amplification of the target molecule.

Amplification of a DNA Target

Amplification of DNA target molecules can proceed only following denaturation of the double-stranded target. This restriction makes the present invention especially useful for the amplification of RNA templates in the presence of excess genomic DNA. In the currently known transcription-based amplification methods, target amplification follows the hybridization of a single primer which is 5'-tailed by the promoter sequence. Hybridization of this primer to double-stranded DNA target may occur due to partial denaturation of the double-stranded target at elevated temperature, 65°C, in the presence of DMSO (included in the amplification mixture for reduction of secondary structure in the template). This process may occur without intentional denaturation of the double-stranded DNA target and will thus reduce the specificity of amplification of an RNA target in samples containing a similar target integrated in DNA molecules contained in the sample, such as genomic DNA.

The discrimination of an RNA gene sequence from a DNA sequence is often required for determination of either free viral components or for determination of gene

expression, as defined by sequence expressed in mRNA species. In the present isothermal, template-switch amplification, the formation of substrate for RNA polymerase is dependent on the hybridization of both TSO and P1 to the same nucleic acid strand, and subsequent template switch in the first primer extension step. Thus, it is highly unlikely that amplification of a DNA sequence could proceed without full denaturation of the double-stranded DNA target.

Refering now to FIG. 2, a sample suspected of containing the specific DNA target is mixed with the appropriate buffer, primers P1 and P2, a TSO, and NTPs (dNTPs and rNTPs), as required for transcription-based amplification. The mixture is heated to 95°C for a short period to allow denaturation of the double-stranded DNA target. At the end of this period, the mixture is incubated at 65°C for a short period and is then incubated at 41°C (or the temperature suitable for activity of the enzymes used for target amplification). Depending on the T_m of the target complementary sequences of the TSO and P1, these oligonucleotides will hybridize to the target at either 65°C or 41°C, to form complex X. P1 hybridizes to the same target DNA strand as the TSO, at a position which is 3' to the TSO hybridization sequence. Following a short incubation at 41°C, the amplification enzymes are added.

As in the case of amplification of an RNA target, the reverse transcriptase extends the P1 primer along the target molecule, up to the site of TSO hybridization. A template switch will occur at this site, as previously described (Patel R. et al., 1996, *supra*). Primer extension then follows along the TSO single-stranded template to produce the complement of the 5'-portion of TSO (which is not complementary to the target). This process results in the production of three-stranded DNA structure XI, which includes a fully functional, double-stranded promoter of the RNA polymerase.

The efficiency of template switch to the TSO is likely to be dependent on the DNA polymerase used and the nature of the target nucleic acid to be amplified. In the case of DNA target sequence it is possible that a DNA dependent DNA polymerase affords a more efficient template switch than a reverse transcriptase. In this case, a DNA dependent DNA polymerase may be included in the amplification reaction mixture. Various DNA dependent DNA polymerase are commercially available and are suitable for use in carrying out the present invention.

The DNA-dependent RNA polymerase will then bind at the promoter site and produce multiple copies of an RNA product IV, which is the same sense as the initial

target sequence. These products will serve as a template for formation of double-stranded DNA products which are the substrate for T7 RNA polymerase, as was described for the amplification of an RNA target. As depicted in FIG. 1b, the process is composed of the following steps: hybridization of the P1 primer to product IV to produce product V; extension of the primer by RT to form RNA/DNA heteroduplex VI; degradation of the RNA template by RNase H to yield a single-stranded DNA product VII; hybridization of primer P2 to the single-strand DNA product; and extension of P2 and the single stranded DNA product by the RT to produce a double stranded DNA product IX, which is in turn a substrate for the RNA polymerase, to produce a plurality of the single-stranded RNA product. IV This process results in further amplification by production of multiple copies of the sense RNA product, as described previously herein.

It should be noted that the incubation temperatures are given as an example and are not limited to the exact temperatures cited. The temperatures will be determined by the requirements for denaturation of the secondary structures of the specific target and the optimum temperature for the amplification enzymes. Moreover, when thermostable enzymes are used, the enzymes can be included in the initial reaction mixture, thus eliminating the need for a separate addition of the amplification enzymes following the initial incubations at elevated temperatures.

It should also be noted, that the RNA products produced by amplification of either RNA or DNA target by the disclosed method are of the same sense as the target nucleic acid strand, in contrast to product of the currently known transcription-based, amplification methods such as NASBA and TMA. Also, the restrictive requirement of formation of a tri-molecular complex, which serves as a unique substrate for template switch during the first primer extension step, results in high specificity of RNA target amplification in the presence of excess double stranded DNA target.

For specific amplification of a DNA target sequence in the presence of RNA targets, usually mRNA, it is desirable to design the amplification primers and TSO to be complementary to sequences of the DNA target strand which are not included in the mRNA. Alternatively, the amplification of a DNA sequence which spans noncoding sequences, i.e., are not present in mRNA, such as intron sequences, may also serve to enhance the specificity of amplification of a DNA sequence.

The formation of single-stranded RNA products renders the amplification process suitable for a wide range of detection processes, including solution phase

homogeneous detection methods, solid phase-based methods, and the various array-based methods.

Branch Migration Inhibition-based procedure for scanning nucleic acid sequences using the strand-switch isothermal amplification

The Branch Migration Inhibition (BMI) method for the detection of sequence alterations in nucleic acid sequences has been described in WO 97/23646, which is incorporated fully herein by reference. The method is based on inhibition of spontaneous strand exchange by branch migration in four-stranded DNA cruciform structures when a test sequence is altered relative to a reference sequence. The BMI substrates are produced by PCR amplification of test and reference DNA sequences using specifically modified primers. Any sequence alterations, such as base substitutions, deletions, and insertions are equally detected, and the method is useful for the detection of sequence alterations in heterozygote and homozygote genotypes. In addition to its potential usefulness for the diagnosis of genetic disease, the method is also useful for the determination of sequence identity required for various applications.

The present invention includes a novel scheme for the formation of substrates for BMI detection of sequence alterations employing the disclosed strand switch isothermal nucleic acid amplification. The method is applicable for detection of sequence alteration in either RNA or DNA target sequences, as required for different applications. In cases where sequence alterations in the expressed gene region are suspected, it may be desirable to use mRNA as initial target molecules, as these are present in excess relative to the genomic sequence. As in any amplification-based method for assessing sequence alteration, it is desirable to limit the extent of *in vitro* amplification, due to potential replication errors introduced by the polymerase used, or the less than perfect fidelity of *in vitro* amplification. The *in vivo* production of multiple copy of mRNA increases the amount of target molecules which in turn reduces the level of *in vitro* amplification required for subsequent analysis. In cases requiring analysis of genomic sequences, the target molecule for analysis will be DNA.

The BMI method for detection of sequence alteration requires the formation of amplification products which are capable, upon denaturation and re-association, of forming partial duplexes which in turn anneal to form four-stranded cruciform structures. When the four-stranded cruciform structures are formed in a mixture of test and

reference amplification products, strand exchange by spontaneous branch migration proceeds if the test and reference amplification products are identical. When the test sequence is different from the reference, branch migration is inhibited, resulting in the formation of stable, detectable four stranded cruciform structures. The utility of branch migration inhibition in the detection of sequence alteration in a test sequence relative to a reference sequence was made possible by the invention of a PCR scheme using specially designed primers.

Referring now to FIG. 3, and as explained more fully in WO 97/23646, in one form of the PCR-based BMI method for detection of sequence alteration, the production of amplification products capable of forming the required partial duplexes is made possible by the use of a mixture of a forward primer P2, and two reverse primers, P1 and P3, for the amplification of both a test and a reference DNA sequence. The two reverse primers have a common 3'-portion, Pa, which is complementary to the target, and 5'-tail sequences, A1 and B1, which are different for the two reverse primers and are not related to the target. Following amplification of the test sequence and the reference sequence, the tailed duplexes form a quadromolecular complex. If a difference exists between the test sequence and the reference sequence, as depicted in FIG 3 as M, strand exchange in the complex ceases, resulting in the formation of a stable complex, C.

This design is adapted for the template switch isothermal amplification by the modification of the primers P1 and P2 shown in FIGS. 1 and 2 and as previously described herein. Referring now to FIG. 4a, primer P1 is replaced by two primers, F1 and F2. Each of these primers has a common 3'-portion, P1, which is complementary to the target, and a 5'-tail, t1 and t2, respectively. The sequences t1 and t2 are different from each other and are not related to the target. In addition, two P2 primers are used, each having the same sequence as described earlier, but are 5'-labeled with two different labels. Biotin and digoxigenin are suiable labels and, as described herein more fully, a number of labels are appropriate. Alternatively, the two labels may be attached to the 5'-end of F1 and F2. When F1 and F2 are labeled only one P2 primer is used. The TSO oligonucleotide is designed similarly to that employed in amplification of either DNA or RNA target, as described above.

The formation of double-stranded DNA products which are capable of formation of the required partial duplexes is described in Figure 4a. Amplification of either DNA

or RNA target sequences proceeds as described above, using substantially equimolar mixtures of the primers F1and F2 and biotin-P2 and Dig.-P2, as well as the TSO. It is also possible to separate the two 5'-labeled P2 primers so that amplification of the test sequence is carried out with P2 primer labeled with one label and the amplification of the reference sequence with the other labeled P2 primer.

As shown in FIG. 4a, amplification of both the test and reference sequences is initiated by combining the sample with a reaction mixture composed of the primers, the TSO, NTPs, and a suitable buffer. FIG. 4a depicts the amplification of either the test or the reference sequence, as only one sequence is shown. However it should be understood that the amplification scheme is identical for both the test and reference nucleic acid sequences. It is preferred that the amplification of the test and reference sequences take place in one vessel. However, amplification in separate vessels is contemplated as part of the present invention.

The mixture is heated to 95°C for denaturation of a DNA target. The mixture is then incubated briefly at 65°C, followed by brief incubation at 41°C, as previously described herein. When the target nucleic acid sequence is an RNA molecule, the initial incubation at 95°C is omitted. A mixture of the amplification enzymes is then added to the reaction mixture and target amplification proceeds at the same temperature. Amplification of a DNA target is initiated by formation of tri molecular complexes XII (test or reference, TSO, and F1) and XIII (test or reference, TSO, and F2). Primer extension by RT and template switch proceed as previously described herein to produce the three-stranded DNA structures XIV and XV, which have a double-stranded promoter for the RNA polymerase. The RNA polymerase produces multiple copies of an RNA transcript, XVI and XVII, of the DNA strands formed by primer extension and template switch. Primers F1 and F2 hybridize to the respective RNA products to yield complexes XVIII and XIX.

Referring now to FIG 4b, primer extension by RT results in formation of RNA/DNA heteroduplexes XX and XXI. The RNA strand of these heteroduplexes is then degraded by RNase H to yield single-strand DNA products XXII and XXIII. The 5' biotin or digoxigenin-labeled primers P2, hybridize to the single-stranded DNA products. Extension of the primer and the single stranded DNA product by RT then follows to produce fully double-stranded DNA products which contain a promoter site for the RNA polymerase at one end, and the double-stranded tails, t1/t1' and t2/t2' at the other end.

The double stranded products are labeled with either one of the two labels used (*e.g.*, biotin and digoxigenin), biotin-or dig-labeled XXIV and biotin- or dig.-labeled XXV. The RNA polymerase binds to the double-stranded products at the promoter site and produces multiple copies of RNA transcripts XXVI and XXVII. These products serve as substrates for formation of double-stranded products as described before. The double-stranded DNA products, biotin-or dig.-labeled XXIV and biotin- or dig.-labeled XXV, are suitable substrates for BMI analysis.

When the target sequence is an RNA molecule, products XIV and XV are composed of both DNA and RNA strands as was previously described for the amplification of an RNA target. The RNA strand of these products is degraded by RNase H. The product contains a double-stranded promoter for the RNA polymerase. Further amplification of the RNA target sequence proceeds as described above for a DNA target sequence.

At the end of the isothermal amplification of the test and reference target sequences, aliquots of the respective amplification reactions are mixed. The RNA products are degraded by RNase, and the mixture is heated to 95°C, to allow denaturation of the double-stranded DNA amplification products. The mixture is then incubated at a lower temperature, 65°C, to allow annealing of the single strands to form complete and partial duplexes. As seen in FIG. 5, partial duplexes comprise double stranded nucleic acid sequences wherein one end thereof has non-complementary oligonucleotide sequences, one linked to each strand of the double stranded molecule. Each non-complementary sequence has 8 to 60, preferably, 10 to 50, more preferably, 15 to 40, nucleotides. Thus, the partial duplex is said to be "tailed" because each strand of the duplex has a single stranded oligonucleotide chain linked thereto.

The degradation of the RNA products prior to BMI analysis is required since these products can compete with the DNA products, as follows. First, the excess RNA product can anneal to the complementary DNA strands, following denaturation of the double-stranded DNA products, thus interfering with formation of partial DNA duplexes. Second, the single-stranded RNA products have a t1' or t2' sequence at their 3'-end, which can compete with the annealing of the partial DNA duplexes, if formed, to form the four-stranded DNA cruciform structures.

Referring now to FIG. 6, the association of the partial duplexes by hybridization of the respective tail sequences forms four-stranded cruciform structures capable of strand exchange via spontaneous branch migration. When the test sequence is identical to the reference sequence, branch migration results in strand exchange, formation of fully double-stranded DNA duplexes, and dissociation of the two labels. When the test sequence is not identical to the reference sequence, branch migration is inhibited, resulting in the formation of stable, four-stranded cruciform structures which are labeled by the two labels. These stable cruciform structures are detectable.

Detection of the stable cruciform structures, indicating sequence alteration in the test sequence relative to a reference sequence, can be carried out using a variety of methods suitable for the detection of the association of the two labels. When the two labels are biotin and digoxigenin, detection can be carried out by EIA. EIA using streptavidin coated microtiter plates and an enzyme anti digoxin monoclonal antibody conjugate as previously described in Lishanski et al. 1996, A homogenous mutation detection method based on inhibition of branch migration, Abstract of the 28th Annual Oakridge Conference on Advanced Analytical Concepts for the Clinical Laboratory, "Tomorrow's Technology Today", p. 15. Homogeneous detection methods are preferred. Various homogeneous detection methods are known, such as the scintillation proximity assay method, the electrochemical luminescence method, and the Luminescent Oxygen Channeling Immunoassay (LOCI) detection method. Performance of BMI/LOCI detection of sequence alteration is shown in WO 97/23646.

A potential advantage of the new procedure for detection of sequence alteration in a test sequence as compared to a reference sequence, in contrast to the PCR-based method, is the relative resistance of the new amplification method to nonspecific priming. Nonspecific PCR priming was shown to lead to formation of stable cruciform structures which are independent of sequence alteration. Unlike the PCR based method, mispriming in the current amplification method cannot lead to the formation of amplification products, in so far as products of mispriming are not expected to undergo the switch of replication template from the target to the TSO. The last step is essential for the formation of the first double-stranded functional promoter for the RNA polymerase. No amplification is possible when this unique product is not formed.

In carrying out the present method, an aqueous medium is employed. Other polar cosolvents may also be employed, usually oxygenated organic solvents of from 1

to 6, more usually from 1 to 4, carbon atoms, including alcohols, ethers and the like. Usually these cosolvents, if used, are present in less than about 70 weight percent, more usually in less than about 30 weight percent.

The pH for the medium is usually in the range of about 4.5 to 9.5, more usually in the range of about 5.5 - 8.5.. In general for amplification, the pH and temperature are chosen and varied, as the case may be, so as to cause, either simultaneously or sequentially, dissociation of any internally hybridized sequences, hybridization of the oligonucleotide primers or TSO, with the target nucleic acid sequence, extension of the primers. Various buffers may be used to achieve the desired pH and maintain the pH during the determination. Illustrative buffers include borate, phosphate, carbonate, Tris, barbital and the like. The particular buffer employed is not critical to this invention but in individual methods one buffer may be preferred over another. The buffer employed in the present methods normally contains magnesium ion (Mg^{2+}), which is commonly used with many known polymerases, although other metal ions such as manganese have also been used. Preferably, magnesium ion is used at a concentration of from about 1 to 20mM, preferably, from about 1.5 to 15mM, more preferably, 3-12mM. The magnesium can be provided as a salt, for example, magnesium chloride and the like. The primary consideration is that the metal ion permit the distinction between different nucleic acids in accordance with the present invention.

The concentration of the nucleotide polymerase is usually determined empirically. Preferably, a concentration is used that is sufficient such that further increase in the concentration does not decrease the time for the amplification by over 5-fold, preferably 2-fold. The primary limiting factor generally is the cost of the reagent.

The amount of the target nucleic acid sequence to be subjected to subsequent amplification using primers in accordance with the present invention may vary from about 1 to 10^{10}, more usually from about 10^3 to 10^8 molecules, preferably at least 10^{-21}M in the medium and may be 10^{-10} to 10^{-19}M, more usually 10^{-14} to 10^{-19}M.

The amount of the oligonucleotide primers and TSO used in the amplification reaction in the present invention will be at least as great as the number of copies desired and will usually be 10^{-9} to 10^{-3} M, preferably, 10^{-7} to 10^{-4} M. Preferably, the concentration of the oligonucleotide primer(s) is substantially in excess over, preferably at least 100 times greater than, more preferably, at least 1000 times greater than, the

concentration of the target nucleic acid sequence. The concentration of the nucleoside triphosphates in the medium can vary widely; preferably, these reagents are present in an excess amount for both amplification and chain extension. The nucleoside triphosphates are usually present in 10^{-6} to 10^{-2}M, preferably 10^{-5} to 10^{-3}M.

As mentioned above, the identity of the target nucleic acid sequence does not need to be known except to the extent to allow preparation of the necessary primers and TSO for carrying out the above reactions. The present invention permits the determination of the presence or absence of a mutation in a nucleic acid sequence in a sample without the need to fully identify the sequence of the nucleic acid. Accordingly, one is able to determine the presence of a mutation in a nucleic acid sequence that is flanked by two sequences of nucleotides for which primers can be made.

Detection

We have described a process for the detection of any sequence alteration in a target molecule relative to a reference sequence, based on amplification and BMI analysis of the amplified sequences. Sequence determination could be directly obtained from the amplification products generated by the disclosed method. Various methods could be employed for sequence determination: Transcription-based sequencing (Sasaki N., *et al, PNAS*, 95:3455-3460 (1998)) can be carried out using the double-stranded DNA product as a substrate. Alternatively, sequencing by hybridization of the single-stranded RNA product to an oligonucleotide array (Gene Chip) could be employed. Other methods which are based on probe hybridization to the single-stranded RNA product could also be used for obtaining sequence information.

The combination of BMI analysis for detection of gene sequence alteration and sequence determination of altered test sequences is most desirable for large-scale testing, such as in screening for genetic abnormalities in which the abnormal state is associated with various gene alterations of a given sequence, as well as other life science applications requiring the assessment of sequence identity.

The detection of two different entities is pertinent to the present invention:

(1) The detection of the single stranded RNA products generated by the isothermal amplification, and

(2) The detection of the stable cruciform complex generated by the BMI analysis of test and reference nucleic acid sequence, if a difference is present between the related sequences.

One means of detecting the RNA product generated by the amplification of the present invention is formation of a trimolecular complex comprising the single stranded RNA product and two oligonucleotide probes. Each of the oligonucleotide probes comprises a sequence, which is complementary to a sequence on the RNA product, and a first or second labels, respectively. The two labels become associated by virtue of both being present within the trimolecular complex, in the presence of the single stranded amplification products. Detection of the association of the two labels in the complex provides for detection of the complex and thus detection of the amplification product.

Similarly, in the present invention one means of detecting the stable cruciform structures, indicating sequence alteration in a test sequence relative to a reference sequence, involves the use of two labels on non-complementary strands of the quadramolecular complex. The two labels become associated by virtue of both being present in the quadamolecular complex if a difference is present between the related sequences. Detection of the two labels in the complex provides for detection of the complex and thus detection of the presence of difference between the two related sequences. Generally, the association of the two labels within the complex is detected.

Detection of the association of two labels in a stable complex provides for detection of either the trimolecular complex or the quadramolecular complexes of the present invention. The association of the labels within the complex may be detected in many ways.

For example, one of the labels can be an sbp member and a complementary sbp member is provided attached to a support. Upon the binding of the complementary sbp members to one another, the complex becomes bound to the support and is separated from the reaction medium. The other label employed is a reporter molecule that is then detected on the support. The presence of the reporter molecule on the support indicates the presence of the complex on the support, which in turn indicates the presence of the mutation in the target nucleic acid sequence. An example of a system as described above is the enzyme-linked immunosorbent assay

(ELISA), a description of which is found in *Enzyme-Immunoassay,* Edward T. Maggio, editor, CRC Press, Inc., Boca Raton, Florida (1980) wherein, for example, the sbp member is biotin, the complementary sbp member is streptavidin and the reporter molecule is an enzyme such as alkaline phosphatase.

Detection of the signal will depend upon the nature of the signal producing system utilized. If the reporter molecule is an enzyme, additional members of the signal producing system would include enzyme substrates and so forth. The product of the enzyme reaction is preferably a luminescent product, or a fluorescent or non-fluorescent dye, any of which can be detected spectrophotometrically, or a product that can be detected by other spectrometric or electrometric means. If the reporter molecule is a fluorescent molecule, the medium can be irradiated and the fluorescence determined. Where the label is a radioactive group, the medium can be counted to determine the radioactive count.

The association of the labels within the complex may also be determined by using labels that provide a signal only if the labels become part of the complex. This approach is particularly attractive when it is desired to conduct the present invention in a homogeneous manner. Such systems include enzyme channeling immunoassay, fluorescence energy transfer immunoassay, electrochemiluminescence assay, induced luminescence assay, latex agglutination and the like.

In one aspect of the present invention detection of the complex is accomplished by employing at least one suspendable particle as a support, which may be bound directly to a nucleic acid strand or may be bound to an sbp member that is complementary to an sbp member attached to a nucleic acid strand. Such a particle serves as a means of segregating the bound target polynucleotide sequence from the bulk solution, for example, by settling, electrophoretic separation or magnetic separation. A second label, which becomes part of the complex, is a part of the signal producing system that is separated or concentrated in a small region of the solution to facilitate detection. Typical labels that may be used in this particular embodiment are fluorescent labels, particles containing a sensitizer and a chemiluminescent olefin (see U.S. Patent No. 5,709,994, the disclosure of which is incorporated herein by reference), chemiluminescent and electroluminescent labels.

Preferably, the particle itself can serve as part of a signal producing system that can function without separation or segregation. The second label is also part of the

signal producing system and can produce a signal in concert with the particle to provide a homogeneous assay detection method. A variety of combinations of labels can be used for this purpose. When all the reagents are added at the beginning of the reaction, the labels are limited to those that are stable to the elevated temperatures used for amplification, chain extension, and branch migration. In that regard it is desirable to employ as labels polynucleotide or polynucleotide analogs having 5 to 20 or more nucleotides depending on the nucleotides used and the nature of the analog. Polynucleotide analogs include structures such as polyribonucleotides, polynucleoside phosphonates, peptido-nucleic acids, polynucleoside phosphorothioates, homo DNA and the like.

In general, uncharged nucleic acid analogs provide stronger binding and shorter sequences can be used. Included in the reaction medium are oligonucleotides or polynucleotide analogs that have sequences of nucleotides that are complementary to the label sequences. One of these oligonucleotides or oligonucleotide analogs is attached to, for example, a reporter molecule or a particle. The other is attached to a primer, either primer F1 or primer F2 and/or P2 or a probe, as a label. Neither the oligonucleotide nor polynucleotide analog attached to the primers, should serve as a polynucleotide polymerase template. This is achieved by using either a polynucleotide analog or a polynucleotide that is connected to the primer by an abasic group. The abasic group comprises a chain of 1 to 20 or more atoms, preferably at least 6 atoms, more preferably, 6 to 12 atoms such as, for example, carbon, hydrogen, nitrogen, oxygen, sulfur, and phosphorus, which may be present as various groups such as polymethylenes, polymethylene ethers, hydroxylated polymethylenes, and so forth. The abasic group conveniently may be introduced into the primer during solid phase synthesis by standard methods.

Under the proper annealing temperature an oligonucleotide or polynucleotide analog attached to a reporter molecule or a particle can bind to its complementary polynucleotide analog or oligonucleotide separated by an abasic site that has become incorporated into the partial duplexes as labels during amplification. If the oligonucleotides or polynucleotides analog become part of a trimolecular or quadramolecular complex, the reporter molecule or particle becomes part of the complex. By using different polynucleotide analogs or oligonucleotide sequences for

labels, two different reporter molecules or particles can become part of the complex. Various combinations of particles and reporter molecules can be used.

When the polynucleotide analog or oligonucleotide label is attached to a probe, as used for the detection of a single stranded amplification product, the polynucleotide analog of oligonucleotide are attached directly at the 5' or the 3' end of the probe sequence which is complementary to the target. In so far as the probes do not serve as substrates for target dependent extension, the attachment of the label sequence to the probe using an abasic spacer is not required. Under proper annealing conditions the labeled probes hybridize to the single stranded amplification product to form a stable complex. When detection of the single stranded product is carried out using two labeled probes, a trimolecular complex is formed, and the two polynucleotide analog or oligonucleotide labels, each attached to the corresponding probe, become associated within the complex. Alternatively, the single stranded amplification product can be detected by using one labeled probe and one labeled primer, each comprising a polynucleotide analog or an oligonucleotide label as described above.

In the present invention one means of detecting the presence of specific nucleic acid sequence involves the isothermal amplification of the invention and detection of the single stranded RNA amplification product. Referring to Figure 7, two oligonucleotide probes, probe 1 and probe 2, and two signal generating particles, a first signal generation particle and a second signal generating particle, are employed for detection of the RNA product. The first probe comprises an oligonucleotide sequence PS1 which is complementary to sequence TS1 on the RNA product and a sequence L1 which is a label and is not complementary to the sequence of the RNA product. The second probe comprises an oligonucleotide sequence PS2 which is complementary to sequence TS2 on the RNA product and a sequence L2 which is a label and is not complementary to the RNA product. Oligonucleotide L1', which is complementary to the label sequence L1, is attached to the first signal generating particle, which may be a chemiluminescer particle. Oligonucleotide L2', which is complementary to the label sequence L2, is attached to the second signal generating particle, which may be a sensitizer. The probes and signal generating particles may be added to the reaction mixture following amplification or may be included in the amplification reaction mixture. The oligonucleotide probes hybridize to the amplification product. Probe 1 bind to the amplification product by hybridization of

sequence PS1 to sequence TS1. Probe 2 similarly binds to the amplification product by hybridization of sequence PS2 to sequence TS2. The trimolecular complex thus formed comprises the single stranded RNA product and the labeled oligonucleotides probes. Binding of the signal generating particles to the trimolecular complex proceeds as described in the following.

Under proper annealing temperature an oligonucleotide or a polynucleotide analog attached to a reporter molecule or a particle can bind to its complementary oligonucleotide or polynucleotide analog attached to the two probes, or to one probe and one primer, as used for detection of the single stranded amplification product. If the oligonucleotide or polynucleotide analog labels become part of the complex, the reporter molecule or particle becomes part of the complex. As mentioned above, by using different oligonucleotide or polynucleotide analog sequences for labels, two different reporter groups or particles can become part of the complex. Various combinations of particles and reporter groups can be used.

The trimolecular complexes or the quadramolecular complexes can also be detected using other ligand/receptor combinations. For example, the two labels attached to the primers or probes may be small molecules such as biotin and digoxigenin and the two corresponding receptors can be streptavidin and anti digoxin antibody. The attachment of the labels to the oligonucleotide primers or probes is carried out by methods known in the art. The receptors are attached to the reporter molecule or particles. Under proper conditions the receptors, for example streptavidin and anti digoxin antibody, attached to a reporter molecule or a particle, bind to the corresponding labels, biotin and digoxin respectively, attached to the primers or probes. If the labels become part of the complex, the reporter molecule or particle becomes part of the complex. The association of the reporter groups or particles is detected. Various combinations of particles and reporter groups can be used.

The particles, for example, may be simple latex particles or may be particles comprising a sensitizer, chemiluminescer, fluorescer, dye, and the like. Typical particle/reporter molecule pairs include a dye crystallite and a fluorescent label where binding causes fluorescence quenching or a tritiated reporter molecule and a particle containing a scintillator. Typical reporter molecule pairs include a fluorescent energy donor and a fluorescent acceptor dye. Typical particle pairs include (1) two latex particles, the association of which is detected by light scattering or turbidimetry, (2) one

particle capable of absorbing light and a second label particle which fluoresces upon accepting energy from the first, and (3) one particle incorporating a sensitizer and a second particle incorporating a chemiluminescer as described for the induced luminescence immunoassay referred to in U.S. Patent No. 5,536,834, which disclosure is incorporated herein by reference. It is also possible to detect particle agglutination due to binding of the two particle species as described in U.S. Patent No. 4,868,104.

Briefly, detection of the complex using the induced luminescence assay as applied in the present invention involves employing a photosensitizer as part of one label and a chemiluminescent compound as part of the other label. If the complex is present the photosensitizer and the chemiluminescent compound come into close proximity. The photosensitizer generates singlet oxygen and activates the chemiluminescent compound when the two labels are in close proximity. The activated chemiluminescent compound subsequently produces light. The amount of light produced is related to the amount of the complex formed.

By way of illustration as applied to the present invention, a particle is employed, which comprises the chemiluminescent compound associated therewith such as by incorporation therein or attachment thereto. The particles have a recognition sequence, usually an oligonucleotide or polynucleotide analog, attached thereto with a complementary sequence incorporated into one of the nucleic acid strands as a first label. Another particle is employed that has the photosensitizer associated therewith. These particles have a recognition sequence attached thereto, which is different than that attached to the chemiluminescent particles. Once the medium has been treated in accordance with the present invention to form a complex, either the trimolecular complex or a quadramolecular complex, the medium is irradiated with light to excite the photosensitizer, which is capable in its excited state of activating oxygen to a singlet state. Because the chemiluminescent compound of one of the sets of particles is now in close proximity to the photosensitizer by virtue of the association of the two labels, the chemiluminescent compound is activated by the singlet oxygen and emits luminescence. The medium is then examined for the presence and/or the amount of luminescence or light emitted, the presence thereof being related to the presence of a stable complex. The presence of the latter indicates the presence and/or amount of the target polynucleotide sequence or the presence of sequence alteration in the target polynucleotide relative to a reference sequence.

As a matter of convenience, predetermined amounts of reagents employed in the present invention can be provided in a kit in packaged combination. A kit can comprise in packaged combination (a) a template switch oligonucleotide, a first primer, and a second primer as described herein. The kit can also include a reference nucleic acid, which corresponds to a target nucleic acid sequence except for the possible presence of a difference such as a mutation, a third and fourth primer, and reagents for conducting an amplification of target nucleic acid sequence prior to subjecting the target nucleic acid sequence to the methods of the present invention. The kit can also include nucleoside triphosphates and a nucleotide polymerase. The kit can further include particles as described above capable of binding to the label on at least one of the primers or probes. The kit can further include members of a signal producing system and also various buffered media, some of which may contain one or more of the above reagents. Preferably, at least all of the above components other than buffer are packaged in a single container.

The relative amounts of the various reagents in the kits can be varied widely to provide for concentrations of the reagents which substantially optimize the reactions that need to occur during the present method and to further substantially optimize the sensitivity of the method in detecting a mutation. Under appropriate circumstances one or more of the reagents in the kit can be provided as a dry powder, usually lyophilized, including excipients, which on dissolution will provide for a reagent solution having the appropriate concentrations for performing a method or assay in accordance with the present invention. Each reagent can be packaged in separate containers or some or all of the reagents can be combined in one container where cross-reactivity and shelf life permit. In a particular embodiment of a kit in accordance with the present invention, the reagents are packaged in a single container. The kits may also include a written description of a method in accordance with the present invention as described above.

EXAMPLES

Materials:

E.coli DNA target was purchased from Sigma and M.tuberculosis DNA target was obtained from Stanford Medical Center.

All the oligodeoxyribonucleotides used were synthesized with specific modifications by Oligo Etc. (Wilsonville, OR) and Operon Technology Inc. (Alameda, CA)

Melting temperatures and priming efficiency for individual oligonucleotides were determined by using the OLIGO computer analysis program.

Molecular weight size markers (50 to 2kb) were purchased from Bio-Rad (Hercules, CA).

Cloned Pfu polymeras was purchased from Stratagene (LaJolla, CA).

Reverse Transcriptase, T7 RNA polymerase, RNase H and an RNase A inhibitor "RNA guard" were purchased from Pharmacia Biotech (Piscataway, NJ).

Ultrapure nucleoside 5'-triphosphate (rNTP) and 2'deoxynucleoside 5'-triphosphates (dNTPs) were purchased as 100 mM solution from Pharmacia Biotech (Piscataway, NJ).

Bovine Serum Albumin (BSA) was purchased from Gibco, Lifetech (Gaithersburg, MD).

RNAse /DNAse-free water from Ambion (Austin, TX).

PCR tubes were purchased from Corning and ISC Bioexpress (Kaysville, UT).

4-10% precast polyacrylamide (native) gels were purchased from Novex (San Diego, CA) and used to analyze the amplication products.

Buffer A: 10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, and 200µg/ml BSA (used for PCR amplification reactions).

Buffer B: 40mM Tris-HCl, pH 8.5, 5mM DTT, 12mM $MgCl_2$, 70mM KCl, 108.8 µg/ml BSA.

Buffer C: 50 mM KCl , 4 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3 , 200 µg/ml BSA (used for LOCI bead suspension).

Abbreviations:

PCR	Polymerase Chain Reaction
NASA	Nucleic Acid Sequence Amplification Analysis
dNTPs	2'-deoxynucleoside 5'-triphosphates
cloned Pfu	Cloned Pyrococus furiosus DNA polymerase
RT	Reverse Transcriptase
RNase H	Cloned Ribonuclease H
RNA guard	RNase A Inhibitor from Human Placenta
TSO	Template Switching Oligonucleotide

BSA	Bovine Serum Albumin
LOCI	Luminescent Oxygen Channeling Immunoassay
DMSO	Dimethyl Sulfoxide

Target Nucleic Acid Sequences

1. E.coli DNA J gene (1358 bp), complete cds. GenBank Accession number M12565

2. M.tuberculosis rpoB gene (3853 bp), partial cds. GenBank Accession number U12205

OLIGONUCLEOTIDE SEQUENCES

Underlined sequences denote sequences complementary to target or product sequences

E.coli DNA J gene

TSO 5' - AAT TCT AAT ACG ACT CAC TAT AGG GAG AGA TCG AGT AGC TCC CTG ACA GTG CAC TGT CAG G GC CGC GGT ACG CTG ATC AAA GAT CC - 3' (Seq. ID No. 1)

Primer 1 5'-CTG TAC GTG GCG TGA CCA AAG AGA - 3' (Seq. ID No. 2)]

Primer 2 5'-AAT TCT AAT ACG ACT CAC TAT AGG G AG AGA TCG AGT AGC TC-3' (Seq. ID No. 3)

Probe 1 5' - TTT TTT TTT TTT TTT TTT TT A ACC AGG TAC ACA GCC GCA GAC TT X - 3' (SEQ. ID NO. 4) (X = C-7 Amino modified)

Probe 2 5' - GTC CGA CCT GTC ATG GTT CTG GT T ACT TAC TTA CTT ACT TAC T X - 3' (Seq. ID No. 5) (X = C-7 Amino modified)

M.tuberculosis rpoB gene

TSO 5' - AAT TCT AAT ACG ACT CAC TAT AGG GAG TTT TCC CAG TCA CAG CGA GCT GAT ATC AGC TCG CC G ACC GTA CGC AGG CGG CGG TTG CCG - 3' (Seq. ID No. 6)

Primer 1 5' - GAT CGG GCA CAT CCG GCC GT - 3' (Seq. ID No. 7)

Primer 2 5' - AAT TCT AAT ACG ACT CAC TAT AGG G AG TTT TCC CAG TCA CA - 3' (Seq. ID No. 8)

Probe 1 5' - TGA ATT GGC TCA GCT GGC TGG TTA CTT ACT TAC TTA CTT ACT X - 3' (Seq. ID No. 9) (X = C-7 Amino modified)

Probe 2 5' - TTT TTT TTT TTT TTT TTT TTG ACA GCG GGT TGT TCT GGT CCA X - 3' (Seq. ID No. 10) (X = C-7 Amino modified)

PREPARATION OF LOCI PARTICLES

C-28 Thioxene:

To a solution of 4-bromoaniline (30g, 174mmol) in dry DMF (200mL) was added 1-bromotetradecane (89.3mL, 366mmol) and N,N-diisopropylethylamine (62.2mL, 357mmol). The reaction solution was heated at 90°C for 16 hr under argon before being cooled to room temperature. To this reaction solution was again added 1-bromotetradecane (45mL, 184mmol) and N,N-diisopropylethylamine (31mL, 178mmol) and the reaction mixture was heated at 90°C for another 15 hr. After cooling, the reaction solution was concentrated *in vacuo* and the residue was diluted with CH_2Cl_2 (400mL). The CH_2Cl_2 solution was washed with 1N aqueous NaOH (2x), H_2O, and brine, was dried over Na_2SO_4 and was concentrated *in vacuo* to yield a dark brown oil (about 110g). Preparative column chromatography on silica gel by a Waters 500 Prep LC system eluting with hexane afforded a yellow oil that contained mainly the product (4-bromo-N,N-di-(tetradeca)-aniline) along with a minor component 1-bromotetradecane. The latter compound was removed from the mixture by vacuum distillation (bp 105-110°C, 0.6mm) to leave 50.2g (51%) of the product as a brown oil. To a mixture of magnesium turnings (9.60g, 395mmol) in dry THF (30mL) under argon was added dropwise a solution of the above substituted aniline product (44.7g, 79mmol) in THF (250mL). A few crystals of iodine were added to initiate the formation of the Grignard reagent. When the reaction mixture became warm and began to reflux, the addition rate was regulated to maintain a gentle reflux. After addition was complete, the mixture was heated at reflux for an additional hour. The cooled supernatant solution was transferred via cannula to an addition funnel and added dropwise (over 2.5 hr) to a solution of anhydrous phenylglyoxal (11.7g, 87mmol) in THF (300mL) at -30°C under argon. The reaction mixture was gradually warmed to 0°C over 1 hr and stirred for another 30 min. The resulting mixture was poured into a mixture of ice water (800mL) and ethyl acetate (250mL). The organic phase was separated and the aqueous phase was extracted with ethyl acetate (3x). The combined organic phases were washed with H_2O (2x), brine and was dried over $MgSO_4$. Evaporation of the solvent gave 48.8g of the crude product as a dark green

oily liquid. Flash column chromatography of this liquid (gradient elution with hexane, 1.5:98.5, 3:97, 5:95 ethyl acetate:hexane) afforded 24.7g (50%) of the benzoin product (LSIMS ($C_{42}H_{69}NO_2$): $[M-H]^+$ 618.6, ^1H NMR (250 MHz, $CDCl_3$) was consistent with the expected benzoin product. To a solution of the benzoin product from above (24.7g, 40mmol) in dry toluene (500mL) was added sequentially 2-mercaptoethanol (25g, 320mmol) and TMSCl (100mL, 788mmol). The reaction solution was heated at reflux for 23 hr under argon before being cooled to room temperature. To this was added additional TMSCl (50mL, 394mmol); and the reaction solution was heated at reflux for another 3 hr. The resulting solution was cooled, was made basic with cold 2.5N aqueous NaOH and was extracted with CH_2Cl_2 (3x). The combined organic layers were washed with saturated aqueous $NaHCO_3$ (2x) and brine, was dried over Na_2SO_4 and was concentrated *in vacuo* to give a brown oily liquid. Preparative column chromatography on silica gel by using a Waters 500 Prep LC system (gradient elution with hexane, 1:99, 2:98 ethyl acetate:hexane) provided 15.5g (60%) of the C-28 thioxene as an orange-yellow oil (LSIMS ($C_{44}H_{71}NOS$): $[M-H]^+$ 661.6, ^1H NMR (250 MHz, $CDCl_3$) was consistent with the expected C-28 thioxene product 2-(4-(N,N-di-(tetradeca)-anilino)-3-phenyl thioxene.

Silicon tetra-t-butyl phthalocyanine

Sodium metal, freshly cut (5.0g, 208mmol), was added to 300mL of anhydrous methanol in a two-liter, 3-necked flask equipped with a magnetic stirrer, reflux condenser, a drying tube and a gas bubbler. After the sodium was completely dissolved, 4-t-butyl-1,2-dicyanobenzene (38.64g, 210mmol, from TCI Chemicals, Portland OR) was added using a funnel. The mixture became clear and the temperature increased to about 50°C. At this point a continuous stream of anhydrous ammonia gas was introduced through the glass bubbler into the reaction mixture for 1 hr. The reaction mixture was then heated under reflux for 4 hr. while the stream of ammonia gas continued. During the course of the reaction, as solid started to precipitate. The resulting suspension was evaporated to dryness (house vacuum) and the residue was suspended in water (400mL) and filtered. The solid was dried (60°C, house vacuum, P_2O_5). The yield of the product (1,3-diiminoisoindoline, 42.2g) was almost quantitative. This material was used for the next step without further purification. To a one-liter, three-necked flask equipped with a condenser and a drying

tube was added the above product (18g, 89mmol) and quinoline (200mL, Aldrich Chemical Company, St. Louis MO). Silicon tetrachloride (11mL, 95mmol, Aldrich Chemical Company) was added with a syringe to the stirred solution over a period of 10 minutes. After the addition was completed, the reaction mixture was heated to 180-185°C in an oil bath for 1 hr. The reaction was allowed to cool to room temperature and concentrated HCl was carefully added to acidify the reaction mixture (pH 5-6). The dark brown reaction mixture was cooled and filtered. The solid was washed with 100mL of water and dried (house vacuum, 60°C, P_2O_5). The solid material was placed in a 1-liter, round bottom flask and concentrated sulfuric acid (500mL) was added with stirring. The mixture was stirred for 4 hr. at 60°C and was then carefully diluted with crushed ice (2000g). The resulting mixture was filtered and the solid wad washed with 100mL of water and dried. The dark blue solid was transferred to a 1-liter, round bottom flask, concentrated ammonia (500mL) was added, and the mixture was heated and stirred under reflux for 2 hr., was cooled to room temperature and was filtered. The solid was washed with 50mL of water and dried under vacuum (house vacuum, 60°C, P_2O_5) to give 12g of product silicon tetra-t-butyl phthalocyanine as a dark blue solid. 3-picoline (12g, from Aldrich Chemical Company), tri-n-butyl amine (anhydrous, 40mL) and tri-n-hexyl chlorosilane (11.5g) were added to 12g of the above product in a one-liter, three-necked flask, equipped with a magnetic stirrer and a reflux condenser. The mixture was heated under reflux for 1.5 hr. and then cooled to room temperature. The picoline was distilled off under high vacuum (oil pump at about 1mm Hg) to dryness. The residue was dissolved in CH_2Cl_2 and purified using a silica gel column (hexane) to give 10g of pure product di-(tri-n-hexylsilyl)-silicon tetra-t-butyl phthalocyanine as a dark blue solid. (LSIMS: $[M-H]^+$ 1364.2, absorption spectra: methanol: 674nm (ε 180,000): toluene 678nm,
^1H NMR (250 MHz, $CDCl_3$): δ: -2.4(m,12H), -1.3(m, 12H), 0.2-0.9 (m, 54H), 1.8(s, 36H), 8.3(d, 4H) and 9.6 (m, 8H) was consistent with the above expected product.

Hydroxypropylaminodextran

(1NH_2/ 7 glucose) was prepared by dissolving Dextran T-500 (Pharmacia, Uppsala, Sweden) (50g) in 150 mL of H_2O in a 3-neck round-bottom flask equipped with mechanical stirrer and dropping funnel. To the above solution was added 18.8g of Zn $(BF_4)_2$ and the temperature was brought to 87°C with a hot water bath. Epichlorohydrin

(350mL) was added dropwise with stirring over about 30 min while the temperature was maintained at 87-88°C. The mixture was stirred for 4 hr while the temperature was maintained between 80°C and 95°C, then the mixture was cooled to room temperature. Chlorodextran product was precipitated by pouring slowly into 3L of methanol with vigorous stirring, recovered by filtration and dried overnight in a vacuum oven.

The chlorodextran product was dissolved in 200mL of water and added to 2L of concentrated aqueous ammonia (36%). This solution was stirred for 4 days at room temperature, then concentrated to about 190mL on a rotary evaporator. The concentrate was divided into two equal batches, and each batch was precipitated by pouring slowly into 2L of rapidly stirring methanol. The final product was recovered by filtration and dried under vacuum.

Hydroxypropylaminodextran (1NH_2/ 7 glucose), prepared above, was dissolved in 50mM MOPS, pH 7.2, at 12.5 mg/mL. The solution was stirred for 8 hr at room temperature, stored under refrigeration and centrifuged for 45 min at 15,000 rpm in a Sorvall RC-5B centrifuge immediately before use to remove a trace of solid material. To 10mL of this solution was added 23.1mg of Sulfo-SMCC in 1mL of water. This mixture was incubated for 1 hr at room temperature and used without further purification.

Chemiluminescer particles (TAR beads)

The following dye composition was employed: 20% C-28 thioxene (prepared as described above), 1.6%1-chloro-9,10-bis(phenylethynyl)anthracene (1-Cl-BPEA) (from Aldrich Chemical Company) and 2.7% rubrene (from (from Aldrich Chemical Company). The particles were latex particles (Seradyn Particle Technology, Indianapolis IN). The dye composition (240-250 mM C-28 thioxene, 8-16 mM 1-Cl-BPEA, and 20-30 mM rubrene) was incorporated into the latex beads in a manner similar to that described in U.S. Patent 5,340,716 issued August 23, 1994 (the '716 patent), at column 48, lines 24-45, which is incorporated herein by reference. The dyeing process involved the addition of the latex beads (10% solids) into a mixture of ethylene glycol (65.4%), 2-ethoxyethanol (32.2%) and 0.1N NaOH (2.3%). The beads were mixed and heated for 40 minutes at 95°C with continuos stirring. While the beads are being heated, the three chemiluminescent dyes were dissolved in 2-ethoxyethanol by heating them to 95°C for 30 minutes with continuous stirring. At the end of both incubations, the dye solution was poured into the bead suspension and

the resulting mixture was incubated for an additional 20 minutes with continuous stirring. Following the 20-minute incubation, the beads were removed form the oil bath and are allowed to cool to 40°C ± 10°C. The beads were then passed through a 43-micron mesh polyester filter and washed. The dyed particles were washed using a Microgon (Microgon Inc., Laguna Hills, CA). The beads were first washed with a solvent mixture composed of ethylene glycol and 2-ethoxyethanol (70%/30%). The beads were washed with 500 ml of solvent mixture per gram of beads. This is followed by a 10 % aqueous ethanol (pH 10-11) wash. The wash volume was 400 ml per gram of beads. The beads were then collected and tested for % solid, dye content, particle size, signal and background generation.

Oligonucleotide Bound Chemiluminescer Particles

The oligonucleotide was immobilized on the surface of the above particles in the following manner. Aminodextran (500 mg) was partially maleimidated by reacting it with sulfo-SMCC (157 mg, 10 mL H_2O). The sulfo-SMCC was added to a solution of the aminodextran (in 40 mL, 0.05 M Na_2HPO_4, pH 7.5) and the resulting mixture was incubated for 1.5 hr. The reaction mixture was then dialyzed against MES/NaCl (2x2L, 10 mM MES, 10 mM NaCl, pH 6.0, 4°C). The maleimidated dextran was centrifuged at 15,000 rpm for 15 minutes and the supernatant collected. The supernatant dextran solution (54 mL) was then treated with imidazole (7 mL of 1.0 M solution) in MES buffer (pH 6.0) and into this stirred solution was added the stained chemiluminescer particles (10 mL of 10mg/mL). After stirring for 10 minutes the suspension was treated with EDAC (7 mmol in 10 mM pH 6.0 MES) and the suspension stirred for 30 minutes. After this time, SurfactAmps® (Pierce) Tween-20 (10%, 0.780 mL) was added to the reaction mixture for a final concentration of 0.1%. The particles were then centrifuged at 15,000 rpm for 45 minutes and the supernatant discarded. The pellet was resuspended in MES/NaCl (pH 6.0, 10 mM, 100 mL) by sonication. Centrifugation at 15,000 rpm for 45 minutes, followed by pellet resuspension after discarding the supernatant, was performed twice. The maleimidated dextran chemiluminescer particles were stored in water as a 10 mg/mL suspension.

Thiolated oligonucleotide (oligonucleotide bearing a 5'-bis(6-hydroxyethyldisulfide) group) (Oligos Etc.) was dissolved in water at a concentration of 0.49 mM. To 116 μL of this solution was added 8.3 μL of 3.5 M sodium acetate, pH

5.3 and 8.9 μL of tris(carboxyethyl)phosphine (20 mM). After 30 minutes incubation at room temperature, 548 μL of cold ethanol was added and the mixture was maintained at about 20°C for 1.5 hour. The precipitated oligonucleotide was recovered by centrifugation for 2 min. at 15,000 rpm in an Eppendorf centrifuge, then dissolved in 37.5 μL of 5mM sodium phosphate, 2 mM EDTA, pH 6.

An aliquot of the maleimidated beads prepared above containing 22 mg beads was centrifuged for 30 min. at about 37,000 g, and the pellet was resuspended in 96 μL of 0.26 M NaCl, 0.05% Tween-20, 95 mM sodium phosphate, and 0.95 mM EDTA, pH7. The thiolated oligonucleotide was added and the mixture was maintained at 37°C for 64 hours under argon. A 10 μL aliquot of sodium thioglycolate was added and incubation was continued for 2 hours at 37°C. Water was added to a total volume of 1 mL, and the beads were recovered by centrifugation, then resuspended in 5 mL of 0.1 M NaCl, 0.17 M glycine, 10 mg/mL BSA, 1 mM EDTA, 0.1% Tween-20, and 0.5 mg/mL Calf thymus DNA (Sigma Molecular Biology grade), pH 9.2. After three hours, the beads were recovered and washed three times by centrifugation, twice in buffer A and once in standard PCR buffer. The product was stored refrigerated in PCR buffer. Buffer A contained 0.1 M Tris base (J.T. Baker Chemical Co.), 0.3 M NaCl (Mallinckrodt), 25 mM EDTA Na_2 H_2O (Sigma Chemical Co.), 0.1% BSA (Sigma Chemical Co.), 0.1% dextran (Pharmacia), HBR-1 (Scantibodies), 0.05% Kathon and 0.01% gentamicin sulfate (GIBCO) prepared by dissolving and adjusting pH to 8.20 with concentrated HCl and made up to 10 L with distilled water.

Sensitizer Particles

The sensitizer beads were prepared placing 600 mL of carboxylate modified beads (Seradyn) in a three-necked, round-bottom flask equipped with a mechanical stirrer, a glass stopper with a thermometer attached to it in one neck, and a funnel in the opposite neck. The flask had been immersed in an oil bath maintained at 94+/-1°C. The beads were added to the flask through the funnel in the neck and the bead container was rinsed with 830 mL of ethoxyethanol, 1700 mL of ethylene glycol and 60 mL of 0.1N NaOH and the rinse was added to the flask through the funnel. The funnel was replaced with a 24-40 rubber septum. The beads were stirred at 765 rpm at a temperature of 94+/-1°C for 40 min.

Silicon tetra-t-butyl phthalocyanine (10.0 g) was dissolved in 300 mL of benzyl alcohol at 60+/-5°C and 85 mL was added to the above round bottom flask through the septum by means of a syringe heated to 120+/-10°C at a rate of 3 mL per min. The remaining 85 mL of the phthalocyanine solution was then added as described above. The syringe and flask originally containing the phthalocyanine was rinsed with 40 mL of benzyl alcohol and transferred to round-bottom flask. After 15 min 900 mL of deionized water and 75 mL of 0.1N NaOH was added dropwise over 40 min. The temperature of the oil bath was allowed to drop slowly to 40+/-10°C and stirring was then discontinued. The beads were then filtered through a 43 micron polyester filter and subjected to a Microgon tangential flow filtration apparatus (Microgon Inc., Laguna Hills, CA) using ethanol:water, 100:0 to 10:90, and then filtered through a 43 micron polyester filter. The beads were then collected and tested for percent solid dye content, particle size, and singlet oxygen generation.

Oligonucleotide Bound Sensitizer Particles

Oligonucleotide bound sensitizer particles were prepared in a manner similar to that described above for oligonucleotide bound to chemiluminescer particles.

Example 1

Detection of E. coli DNAJ target sequence by NASBA/LOCI

A schematic description of formation of detectable complex comprising the RNA amplification product and two labeled probes is shown in FIG. 7. Probe 1 and probe 2 comprise a 5' sequence which is complementary to two site on the product, PS1 of probe 1 is complementary to sequence TS1 on the RNA product and sequence PS2 of probe 2 is complementary to sequence TS2 on the RNA product. The two probes further comprise a label L1 and L2, respectively, at their 5' or 3' end. Labels L1 and L2 may be reporter molecules such as biotin and digoxigenin, or may comprise a nucleic acid sequences which are not related to the amplification product or the target. The tri molecular complex formed by the hybridization of the RNA product and the two probes is detectable by numerous method known in the art depending upon the labels chosen.

FIG. 7 depicts the formation of a complex between the tri molecular complex and two signal generating particles, for example a chemiluminescer particle and a sensitizer particle as used in LOCI. When the L1 and L2 labels are oligonucleotide

sequences, complementary oligonucleotide sequences are attached to the respective particles, to enable binding of the two particles to the respective probe label. Thus, oligonucleotide L1' which comprises a sequence complementary to L1 is attached to the first particle, and oligonucleotide L2' which comprises a sequence complementary to L2 is attached to the second particle. The signal-generating complex is formed by hybridization of the two probes to the RNA product and binding of the particles to the complex by hybridization of L1 to L1' and L2 to L2'.

In a specific example, 13.0 µl of the reaction mixture (containing 1 mM of each dNTPs and 2 mM each of rATP, rUTP, rCTP and 1.5 mM rGTP, 0.5 mM rITP, 250 nM of primer 1 and 2 and 50 nM of TSO, DMSO 15% (U/U), in Buffer B) was aliquoted to individual PCR tubes in an 8 tube-strip. 2 µl sample containing E. coli genomic DNA was then added to each tube, followed by 15 µl mineral oil.

The mixture was incubated at 95°C for 5 min., cooled down to 41°C and incubated for 5 min. 5.0 µl enzyme mixture (RT 6.4 u, T7 RNA polymerase 32 u, RNase H 0.08 u, and RNA guard 12 u, in 30% glycerol (v/v) in 10 mM Tris pH 8.5) was then added to each tube and the reactions were further incubated at 41°C for 90 min.

LOCI detection of the amplification products was carried out as follows: 45 µl of the combined detection reagents (containing 12.5 nM of probe 1 and probe 2, and 2.5 ug of the acceptor and sensitizer oligonucleotide coated LOCI beads in Buffer C) were added to individual tubes and 5 µl amplification products (amplification reaction mixture with or without target) or water were then added. The detection reaction mixtures were incubated at 65°C for 2 min, 50°C for 15 min and 37°C for 30 min.

LOCI signals were obtained using a LOCI strip-reader by irradiation for 1 sec. and read for 1 sec. for a total of 3 cycles. The results are summarized in Table 1.

Table 1

Amplification and detection of E. coli target Using NASBA/LOCI

E.coli DNA (molecules)	RLU 1	RLU 2	RLU 3
0	25702	4458	17124
10	22120	3934	19870
100	11328	5796	19700
1000	128594	20500	22976
10000	6312560	58581622	2380320

As shown in Table 1, the detection limit for amplification of the DNAJ gene sequence using RT, T7 DNA dependent RNA polymerase and RNase H, is about 1000 molecules per reaction. It is likely that further optimization of this amplification scheme could enhance sensitivity limit. Optimization of this procedure might be achieved by selecting a DNA polymerase which provides high efficiency of template switching, as shown in the following example.

Example 2

Improved Amplification of DNA Nucleic acid Target By the Addition Of Pfu DNA Polymerase

Reaction mixtures were assembled as described in Example 2. The reaction mixtures were incubated at 95^{0}C for 4 min., cooled down to 65^{0}C and incubated for 2 min. Pfu polymerase (1 u) was added and the reaction mixture was further incubated at this temperature for 5 min. The reactions mixture were then cooled down to 41^{0}C, and 5 ul of the enzyme mixture (as in Example 1) was added. The reaction mixtures were further incubated as in Example 1.

LOCI detection of NASA amplification products was carried out as in Example 1. The results are summarized in Table 2.

Table 2

NASA of E. coli DNA gene target with or without Pfu DNA polymerase added at 65°C

E.coli DNA	+Pfu @ 65°C	-Pfu
0	5620	4458
10	4091	3934
100	15796	5796
1000	5970710	20748
10000	6994281	585816
100000	6396661	6534551

As shown in Table 2, a ten fold increase in the sensitivity of amplification and detection of the target nucleic acid is achieved by this modification, as compared to amplification performed without the addition of Pfu, to reach a detection limit of about

100 molecules. It is likely that Pfu affords a higher efficiency of primer extension and target switch.

Example 3

Effect of Incubation Temperature For Pfu Catalyzed Primer Extension and Target Switch Step

Reaction mixtures were assembled as described in Example 1. The reaction mixtures were incubated at 95°C for 4 min., cooled down to either 50°C or 41°C and incubated for 2 min. Pfu polymerase (1 u) was added to the reaction mixture and the mixtures were incubated for 5 minutes. When Pfu polymerase was added at 50°C, the reaction mixtures were cooled to 41°C and incubated for 5 min., before addition of 5 ul of the enzyme reaction mixture as in Example 1. When Pfu polymerase was added at 41°C, the enzyme reaction mixture was add after 5 min. incubation at this temperature. In another case Pfu polymerase was mixed with the enzyme reaction mixture and the reactions were carried out as in Example 1. LOCI detection of the amplification products was the same as in previous examples.

The effect of incubation temperature for the first primer extension and target switch step, and the efficiency of Pfu polymerase as a separate reagent or as a combined reagent with the three other amplification enzyme, was assessed using two target nucleic acids, the DNAJ gene of E. coli and the rpoB gene of M. tuberculosis. The results of NASA amplification of these genomic nucleic acid targets and LOCI detection of the amplification products are summarized in Table 3 and Table 4, respectively.

Table 3

NASA amplification of E. coli DNAJ Target Sequence With or Without Pfu
(as a separate reagent or a combined reagent added at 41°C)

E.coli DNA	+Pfu @50°C	+Pfu @41°C	+Pfu+3Enzs @41°C	-Pfu
0	16324	13974	12596	8244
10	34834	48748	18352	18854
100	247676	71638	12426	8378
1000	6861391	5379950	5374130	65806
10000	8670282	8227150	8563440	813490

Table 4

NASA Amplification of M. tuberculosis rpoB gene sequence Using TSO1 With or Without Pfu (as a separate reagent or a combined reagent @ 41°C)

M.tuberculosis DNA	+Pfu @50°C	+Pfu @41°C	+Pfu & 3 Enzs @41°C
0	18672	16104	15058
10	19196	21336	40856
100	279942	53922	22892
1000	6421010	6773660	4715910
10000	8435305	7572430	8366991

As shown in Tables 3 and 4, the efficiency of NASA amplification of DNA nucleic acid targets is improved when the first primer extension and target switch step is carried out by Pfu DNA polymerase at either 50^0C or 41^0C , and Pfu polymerase may be added to the amplification enzyme mixture as a single reagent. Detection limit of 10 to 100 molecules was successfully demonstrated using the two genomic DNA targets.

The above discussion includes certain theories as to mechanisms involved in the present invention. These theories should not be construed to limit the present invention in any way. The above description and examples fully disclose the invention including preferred embodiments thereof. Modifications of the methods described that are obvious to those of ordinary skill in the art such as molecular biology and related sciences are intended to be within the scope of the claims.

What is claimed is:

1. A method for producing multiple copies of a target polynucleotide sequence comprising:

subjecting a mixture of the target polynucleotide sequence, a first oligonucleotide primer, a template switch oligonucleotide, and reagents sufficient for conducting an amplification of said target polynucleotide sequence, to conditions sufficient for amplifying the target nucleic acid sequence.

2. The method of claim 1 wherein the template switch oligonucleotide comprises a nucleotide sequence having a 3' region capable of hybridizing to the target sequence and a 5' region which does not hybridize to the target sequence.

3. The method according to claim 2 wherein said 5' region of said template switch oligonucleotide includes a propromoter sequence of a DNA dependent RNA polymerase.

4. The method according to claim 3 wherein said 5' region of said template switch oligonucleotide includes a nucleotide sequence substantially homologous to the target sequence located 3' of said propromoter sequence.

5. The method according to claim 4 wherein said 5' region of said template switch oligonucleotide further comprises a sequence unrelated to the target sequence located between said sequence substantially homologous to the target and said propromoter sequence.

6. The method according to claim 5 wherein said sequence homologous to the target sequence is immediately 5' to said 3' region complementary to the target sequence.

7. The method according to claim 1 wherein the conditions for amplifying said target polynucleotide sequence are isothermal.

8. The method according to claim 1 further comprising the addition of a second oligonucleotide primer.

9. The method according to claim 8 wherein said second primer is not target dependant.

10. The method according to claim 8 wherein said second primer includes a nucleotide sequence substantially homologous to said sequence unrelated to target sequence on said template switch oligonucleotide.

11. The method according to claim 8 wherein said second primer includes the sequence of a propromoter of a DNA dependent RNA polymerase.

12. The method according to claim 1 wherein the target is DNA.

13. The method according to claim 1 wherein the target is RNA.

14. The method according to claim 12 further comprising the step of denaturation of said DNA target.

15. The method of claim 1 wherein the reagents sufficient for conducting an amplification include a polynucleotide polymerase, deoxynucleoside triphosphates, and ribonucleoside triphosphates.

16. A method for producing multiple copies of a target nucleic acid sequence comprising:

hybridizing a first primer and a template switch oligonucleotide to the target sequence; extending said first primer along the target and along the 5' portion of said template switch oligonucleotide to form a first extension product; transcribing said first extension product to produce multiple copies of a transcription product having a sequence homologous to the target sequence.

17. The method of claim 16 further comprising:

hybridizing said first primer to said transcription product; extending said first primer along said transcription product to form a second extension product; degrading said transcription product; hybridizing a second primer to said second extension product; extending said second primer to form a third extension product; extending said second extension product to produce a double stranded DNA product with said

third extension product; transcribing said double stranded DNA product to produce multiple copies of a said transcription product.

18. The method of claim 16 wherein the template switch oligonucleotide comprises a nucleotide sequence having a 3' region capable of hybridizing to the target sequence and a 5' region which does not hybridize to the target sequence.

19. The method according to claim 18 wherein said 5' region of said template switch oligonucleotide includes a propromoter sequence of a DNA dependent RNA polymerase.

20. The method according to claim 19 wherein said 5' region of said template switch oligonucleotide includes a nucleotide sequence substantially homologous to the target sequence located 3' of said propromoter sequence.

21. The method according to claim 20 wherein said 5' region of said template switch oligonucleotide further comprises a sequence unrelated to the target sequence located between said sequence substantially homologous to the target and said propromoter sequence.

22. The method according to claim 21 wherein said sequence homologous to the target sequence is immediately 5' to said 3' region complementary to the target sequence.

23. The method according to claim 16 wherein the conditions for amplifying said target polynucleotide sequence are isothermal.

24. The method according to claim 17 wherein said second primer is not target dependant.

25. The method according to claim 17 wherein said second primer includes a nucleotide sequence substantially homologous to said sequence unrelated to target sequence on said template switch oligonucleotide.

26. The method according to claim 17 wherein said second primer includes the sequence of a propromoter of a DNA dependent RNA polymerase.

27. The method according to claim 16 wherein the target is DNA.

28. The method according to claim 16 wherein the target is RNA.

29. The method according to claim 27 further comprising the step of denaturation of said DNA target.

30. In an isothermal method for amplifying a target polynucleotide sequence, the improvement comprising: using a template switch oligonucleotide and a primer which is not target dependent.

31. A method for detecting the presence of a difference between a target nucleic acid sequence and a reference nucleic acid sequence comprising:

amplifying the target sequence and the reference sequence using a template switch oligonucleotide, a first primer, a second primer, and a third primer; said first primer and said second primer having common 3' sequences which are complementary to the target and the reference, said first and second primers further having 5' tail sequences which are not complementary to the target sequence, the reference sequence, and each other;

wherein either:

(a) said first primer has a first label and said second primer has a second label, or

(b) said third primer includes a mixture of said third primer having a first label and said third primer having second label;

forming a quadromolecular complex including said reference and the target sequences in double stranded form; said complex having at least one pair of non complementary strands and each of the said non complementary strands in the said complex having a label,

subjecting the complex to strand exchange conditions, wherein if no difference is present between said test and reference sequences strand exchange continues to completion, and wherein if a difference between said reference and test sequences is present strand exchange in said complex ceases, and

detecting the association of said labels within said complex, the association thereof being related to the presence of said difference.

32. The method according to claim 31 wherein said first label and said second label are independently selected from the group consisting of oligonucleotides, enzymes, dyes, fluorescent molecules, co-enzymes, enzyme substrates, radioactive groups, small organic molecules, polynucleotide sequences, polynucleotide analog sequences and solid surfaces.

33. The method of claim 31 wherein said difference is a mutation.

34. The method of claim 31 wherein said complex comprises a Holliday junction.

35. The method of claim 31 wherein the amplification of said target sequence and said reference sequence is isothermal.

36. The method according to claim 31 wherein said difference is a single nucleotide polymorphism.

37. The method according to claim 31 wherein said third primer includes the sequence of a propromoter of a DNA dependent RNA polymerase

38. The method according to claim 31 wherein the target is DNA.

39. The method according to claim 31 wherein the target is RNA.

40. The method according to claim 38 further comprising the step of denaturation of said DNA.

41. A method of detecting the presence of a difference between a target nucleic acid sequence and a reference nucleic acid sequence comprising:

combining the target sequence, a first primer, a second primer, a third primer and a fourth primer, a template switch oligonucleotide, and reagents sufficient to amplify the target sequence;

combining in a separate vessel the reference sequence, a first primer, a second primer, a third primer and a fourth primer, a template switch oligonucleotide, and reagents sufficient to amplify the reference sequence;

said first primer and said second primer each having a 3' region which is complementary to the target sequence and the reference sequence and a 5' tail which

is not complementary to the target sequence and the reference sequence, said 5' tail of said first primer being non-complementary to said 5' tail of said second primer;

said template switch oligonucleotide having a 3' region which is complementary to the target sequence and the reference sequence, and a 5' region including a sequence unrelated to the target and the reference sequences; said 5' region further including a propromoter of a DNA dependent RNA polymerase.

said third primer having a first label and said fourth primer having a second label, said third primer and said fourth primer having substantially the same nucleic acid sequences, and said third and fourth primer nucleic acid sequences being substantially homologous to said 5'sequence of the template switch oligonucleotide which is unrelated to target or reference nucleic acid sequences,

forming from said target sequence a tailed target partial duplex of two nucleic acid strands, one of said target strands having said first label or said second label, and each of said strands having non-complementary oligonucleotide tails;

forming from said reference sequence a tailed reference partial duplex of two nucleic acid strands, one of said reference strands having said first label or said second label, and each of said strands having non-complementary oligonucleotide tails.

forming a quadromolecular complex comprising said tailed target duplex and said tailed reference duplex in double stranded form, wherein said complex includes at least one pair of non-complementary strands and each of said non-complementary strands within said complex has a label;

subjecting the complex to strand exchange conditions, wherein if no difference is present between the target and the reference sequences, strand exchange continues to completion, and wherein if a difference between said reference and test sequences is present, strand exchange in said complex ceases, and

detecting the association of said labels within said complex, the association thereof being related to the presence of the difference.

42. The method according to claim 41 wherein said first label and said second label are independently selected from the group consisting of oligonucleotides, enzymes, dyes, fluorescent molecules, co-enzymes, enzyme substrates, radioactive groups, small organic molecules, polynucleotide sequences, polynucleotide analog sequences, and solid surfaces.

43. The method of claim 41 wherein said difference is a mutation.

44. The method of claim 41 wherein said target sequence is RNA or DNA.

45. The method of claim 41 wherein said difference is a single nucleotide polymorphism.

46. The method of claim 41 wherein said complex comprises a Holliday junction.

47. The method of claims 41 wherein the target sequence, the reference sequences, said primers, said template switch oligonucleotide and said reagents are combined in the same reaction vessel.

48. A kit for producing multiple copies of a target nucleic acid, said kit comprising in packaged form:

(a) a template switch oligonucleotide having the sequence of a propromoter of a DNA dependent RNA polymerase;

(b) a first primer extendable along said target, and said template switch oligonucleotide.

49. The kit according to claim 48 further comprising a second primer have the sequence of a propromoter of a DNA polymerase and a sequence to a portion of the template switch oligonucleotide which is substantially adjacent to said propromoter sequence on said template switch oligonucleotide.

50. The kit according to claim 48 further comprising a first probe, a second probe, a first signal generating particle and a second signal generating particle.

51. A kit for detecting the presence of a difference between a reference nucleic acid sequence and a target nucleic acid sequence comprising

(a) a reference nucleic acid sequence;

(b) a template switch oligonucleotide having a sequence of a propromoter of an RNA polymerase;

(c) a first primer and a second primer each comprising a common 3'-end sequence which is complementary to said target and reference nucleic acid sequence, and a 5'-end sequence which is not complementary to

said target or reference nuclei acid sequence or to each other, extendable along said target, said reference and said template switch oligonucleotide;

(d) a third primer having the sequence of a propromoter of the RNA polymerase and a sequence homologous to a portion of the template switch oligonucleotide which is substantially adjacent to said propromoter sequence on said template switch oligonucleotide;

wherein either:

said first primer has a first label and said second primer has a second label, or said third primer is a mixture of said third primer with a first label and said third primer with a second label.

FIG. 1a

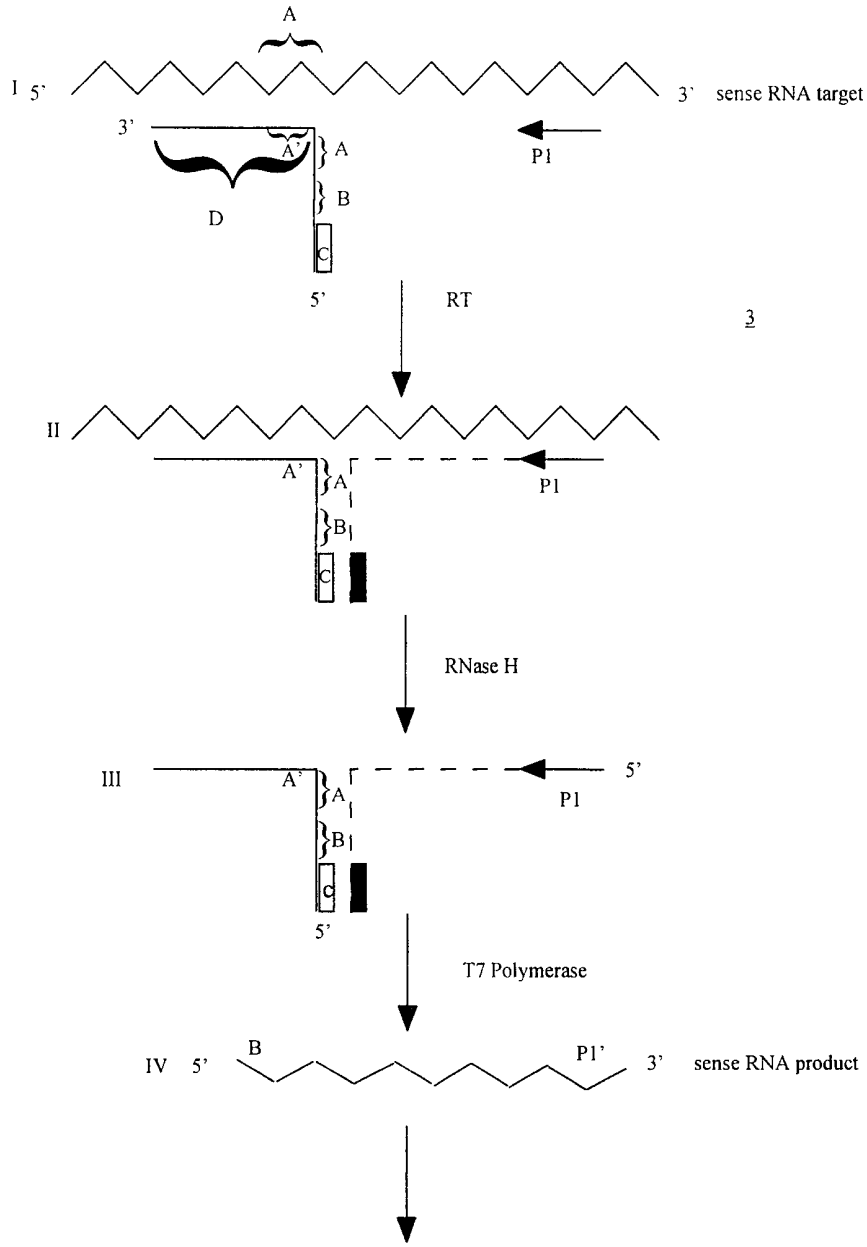

A
I 5'
3' sense RNA target
3'
A'
A
P1
B
D
C
5'
RT
3
II
A'
A
P1
B
C
RNase H
III
A'
5'
A
P1
B
C
5'
T7 Polymerase
IV 5'
B
P1'
3'
sense RNA product

FIG. 1b

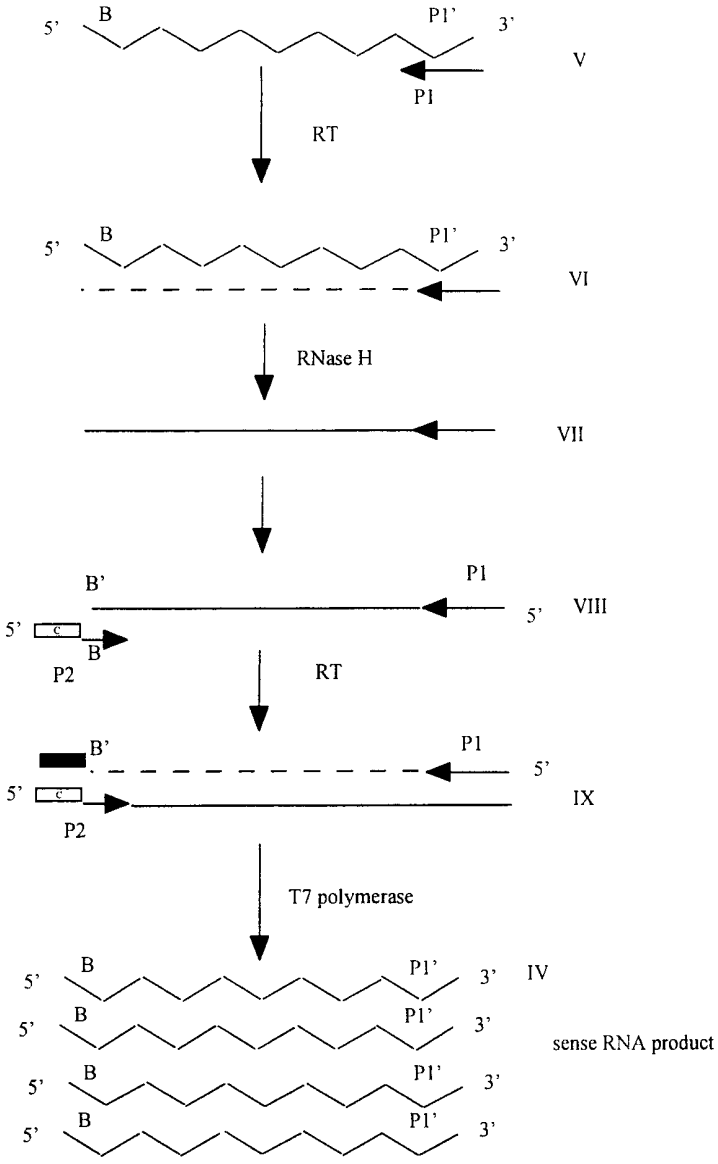
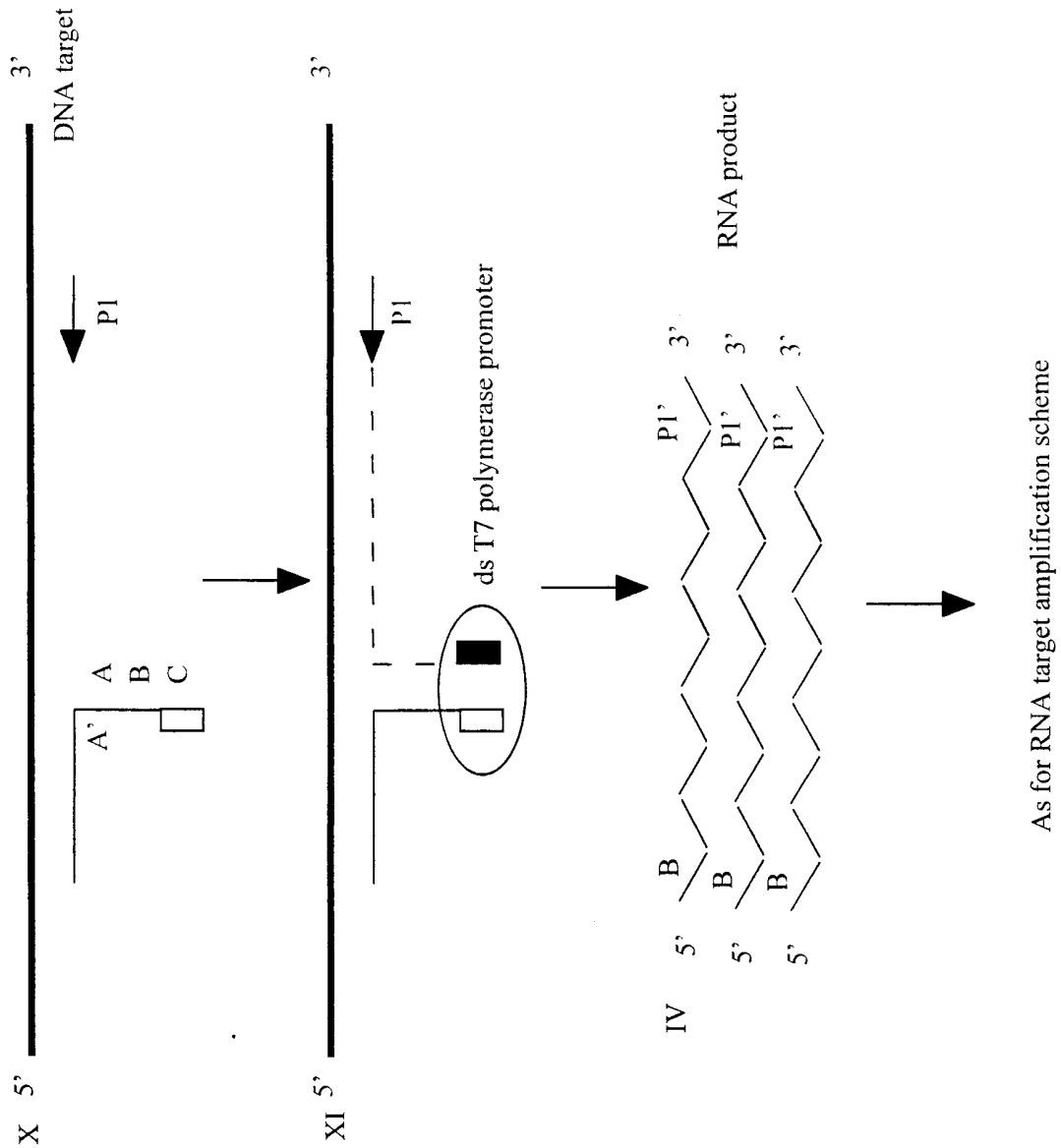

5'
B
P1'
3'
V
P1
RT
VI
RNase H
VII
B'
P1
VIII
C
P2
IX
T7 polymerase
IV
sense RNA product

FIG. 2


3'
DNA target
P1
A
B
C
A'
X 5'
3'
P1
ds T7 polymerase promoter
XI 5'
P1'
3'
RNA product
B
IV 5'
As for RNA target amplification scheme

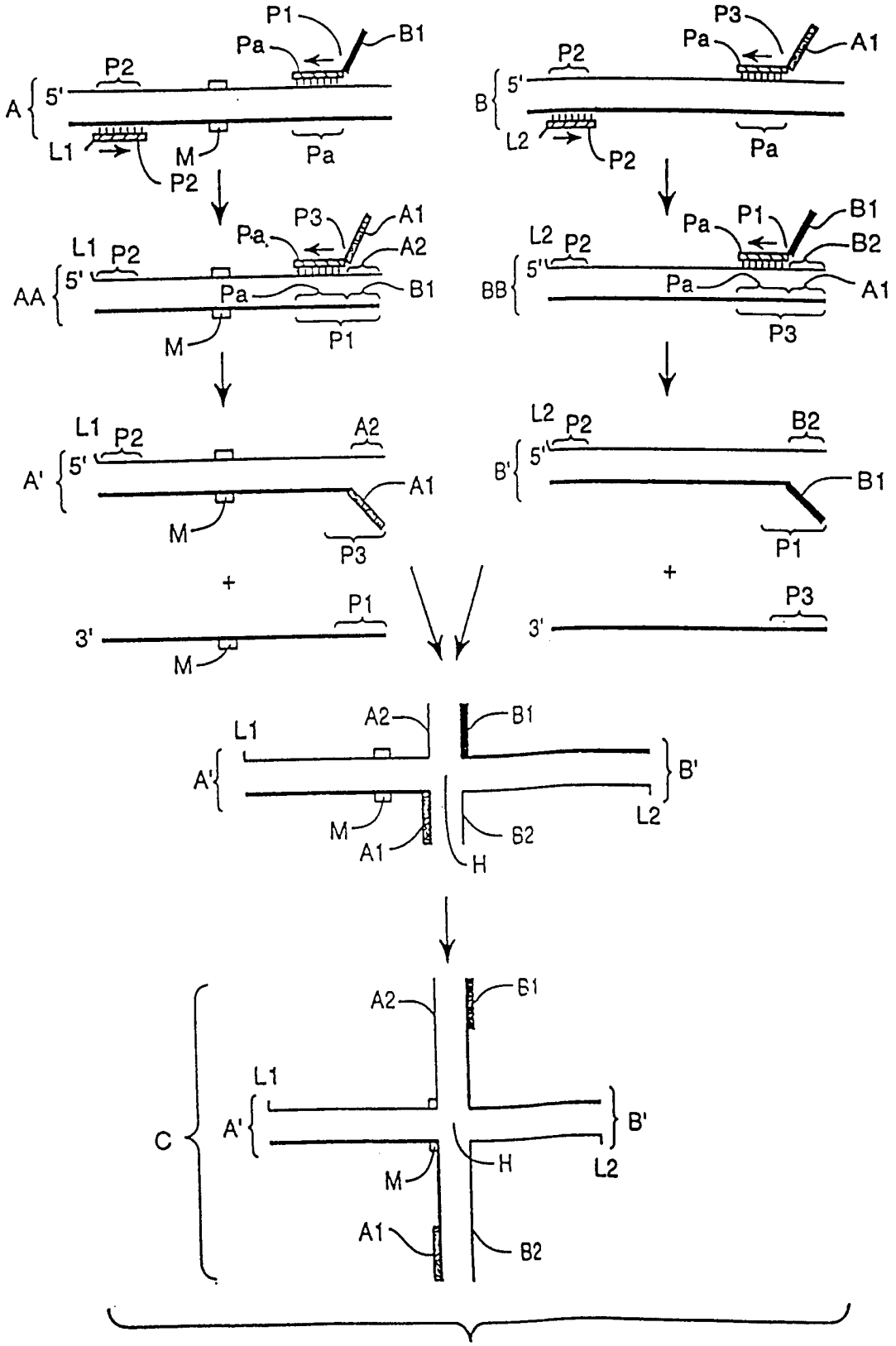

P1
Pa
B1
P2
A
5'
L1
M
P2
Pa
P3
Pa
A1
P2
B
5'
L2
P2
Pa
L1 P2
P3
A1
Pa
A2
AA
5'
Pa
B1
M
P1
L2 P2
Pa
P1
B1
BB
5'
B2
Pa
A1
P3
L1 P2
A2
A'
5'
A1
M
P3
L2 P2
B2
B'
5'
B1
P1
+
P1
3'
M
+
P3
3'
L1
A2
B1
A'
B'
M
A1
H
B2
L2
A2
B1
L1
C
A'
B'
M
H
L2
A1
B2

FIG._3

FIG. 4a

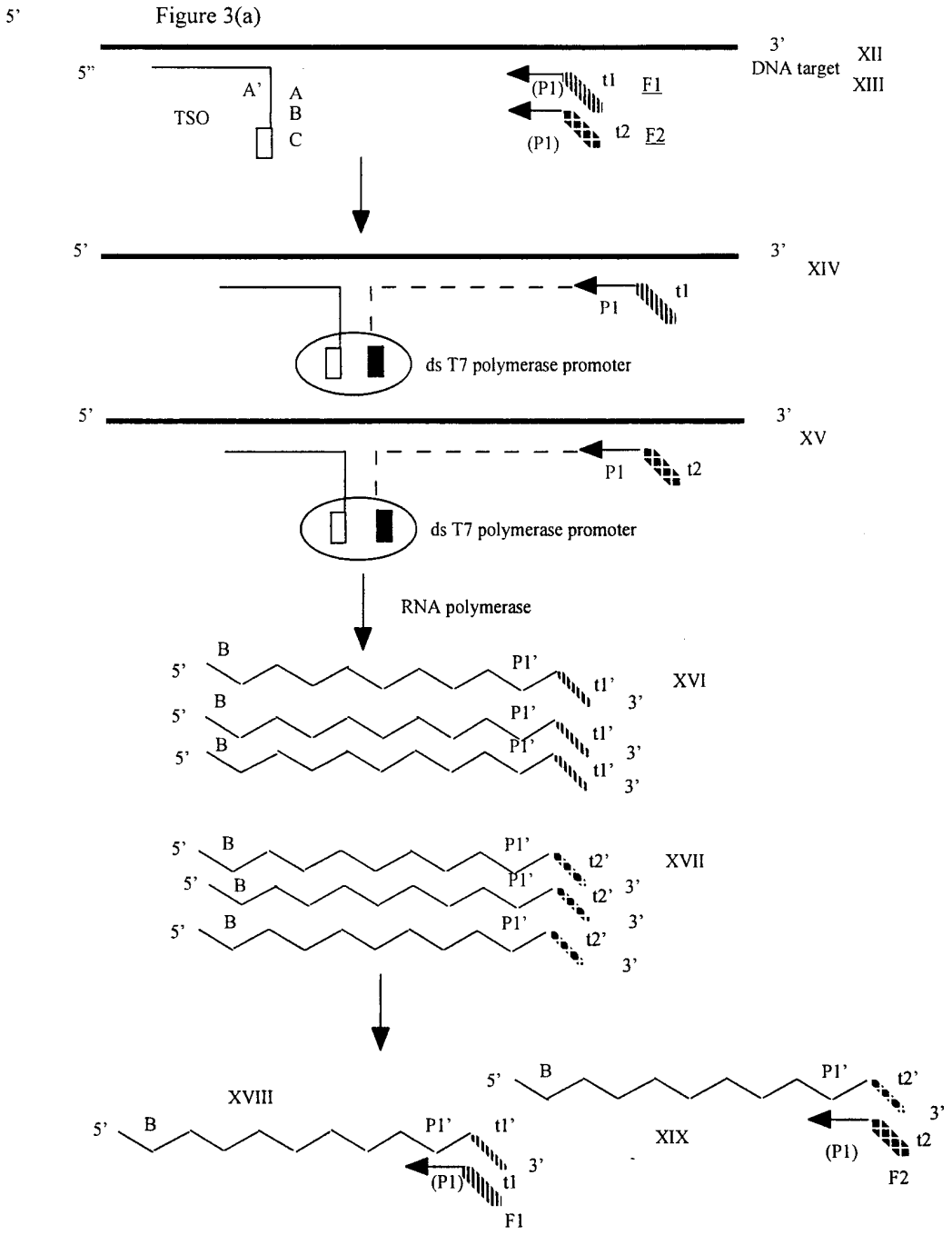

5'
Figure 3(a)
3'
DNA target
XII
XIII
5"
A'
A
B
C
TSO
(P1)
t1
F1
(P1)
t2
F2
5'
3'
XIV
P1
t1
ds T7 polymerase promoter
5'
3'
XV
P1
t2
ds T7 polymerase promoter
RNA polymerase
B
P1'
t1'
XVI
3'
t2'
XVII
XVIII
XIX
F1
F2

FIG. 4b

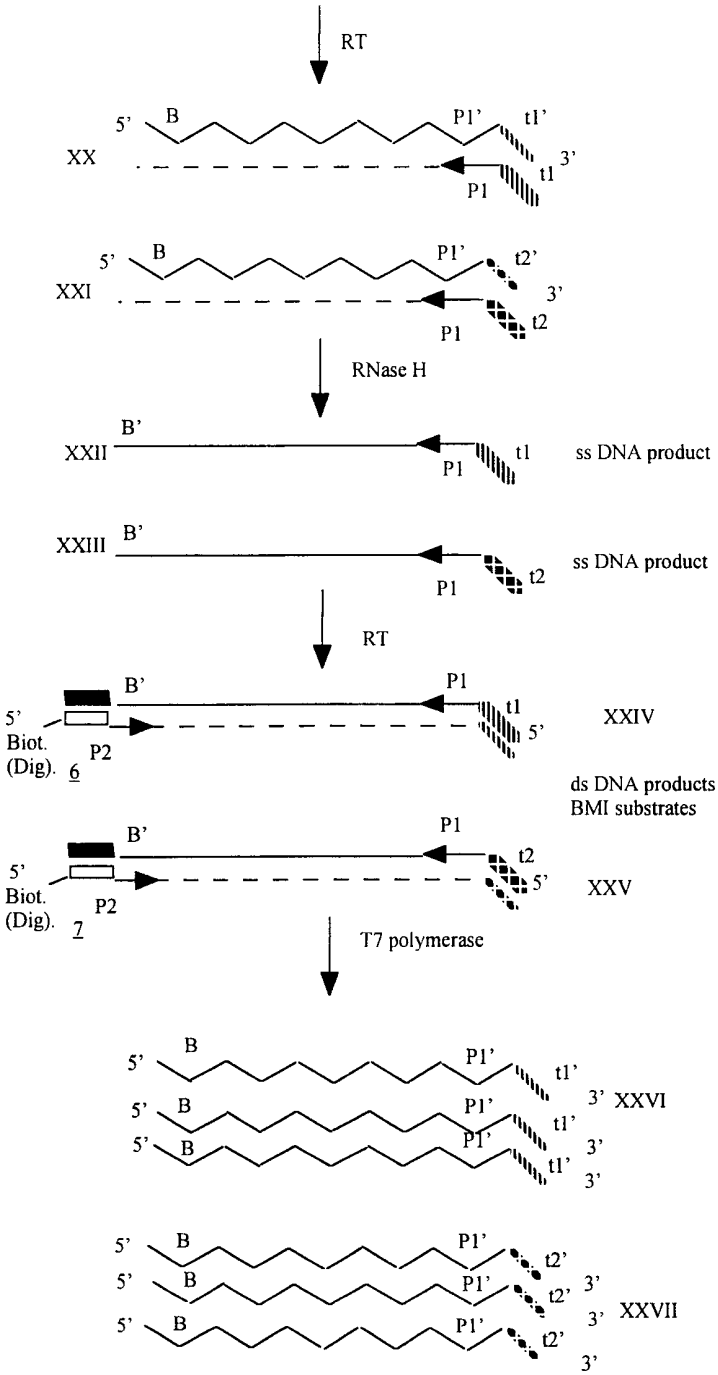

RT
5' B P1' t1'
XX 3'
t1
P1
5' B P1' t2'
XXI 3'
P1 t2
RNase H
B'
XXII t1 ss DNA product
P1
XXIII B' ss DNA product
P1 t2
RT
B' P1
5' t1
Biot. P2 5' XXIV
(Dig). 6
ds DNA products
BMI substrates
B' P1
5' t2
Biot. P2 5' XXV
(Dig). 7
T7 polymerase
5' B P1' t1' 3' XXVI
5' B P1' t1' 3'
5' B P1' t1' 3'
5' B P1' t2'
5' B P1' t2' 3'
5' B P1' t2' 3' XXVII
3'

FIG. 5

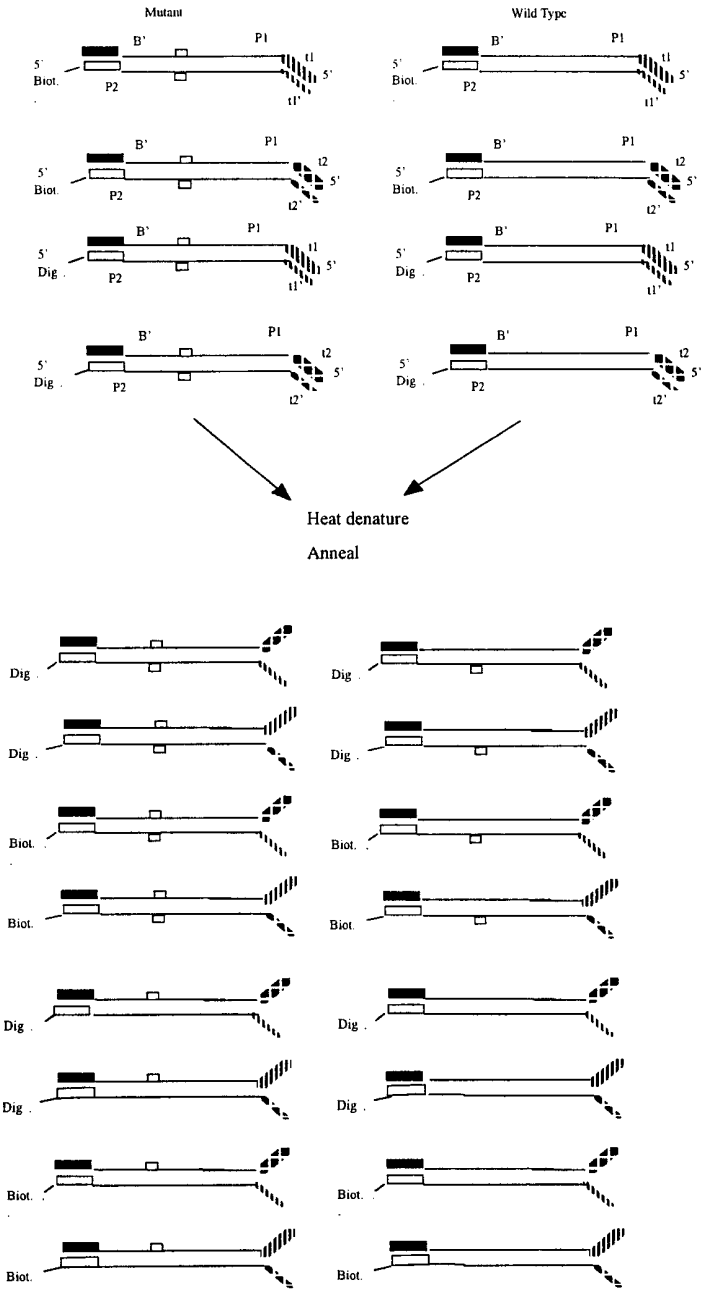

Mutant
Wild Type
B'
P1
P2
5'
Biot.
Dig
t1
t1'
t2
t2'
Heat denature
Anneal

FIG. 6

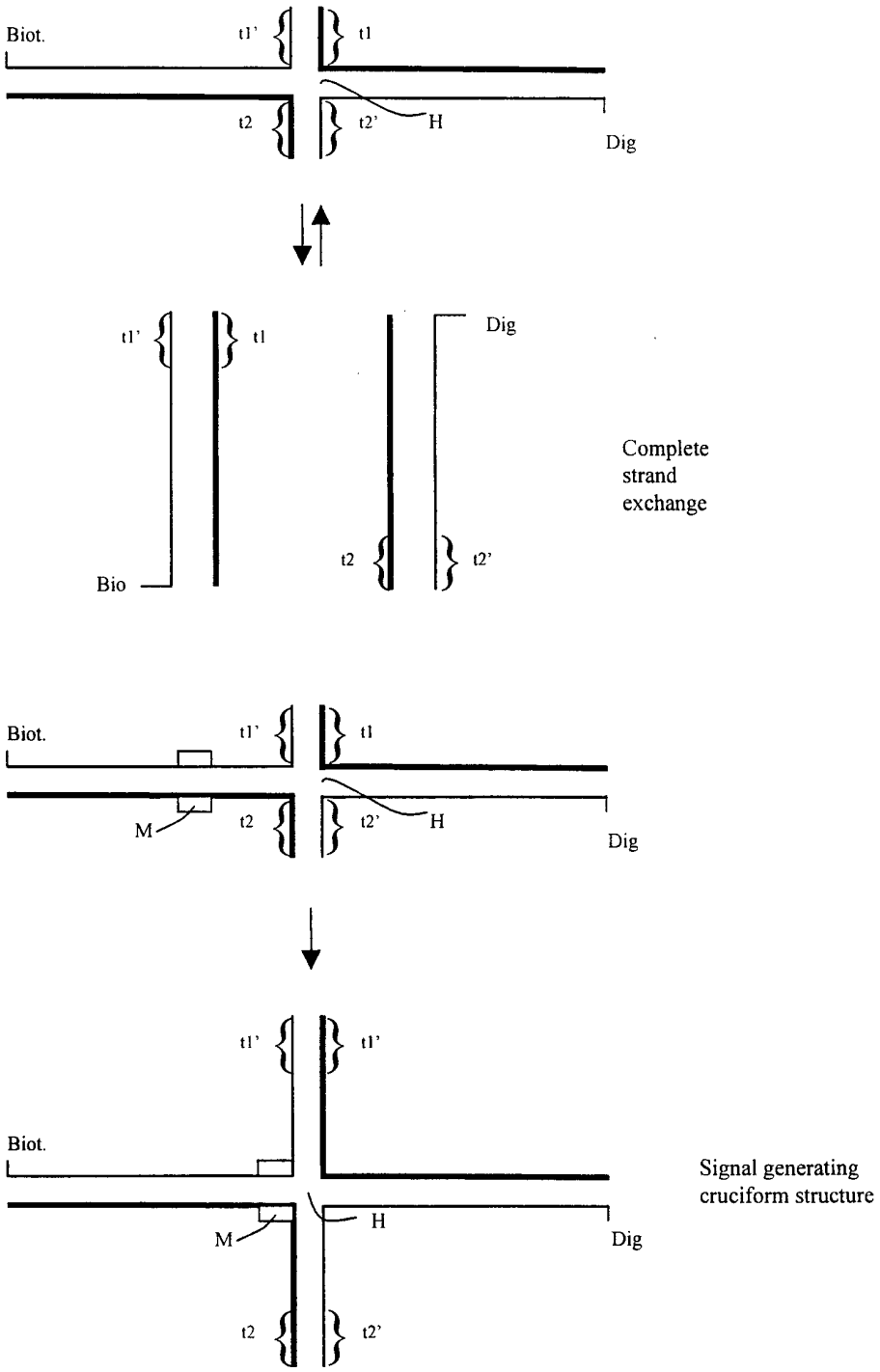
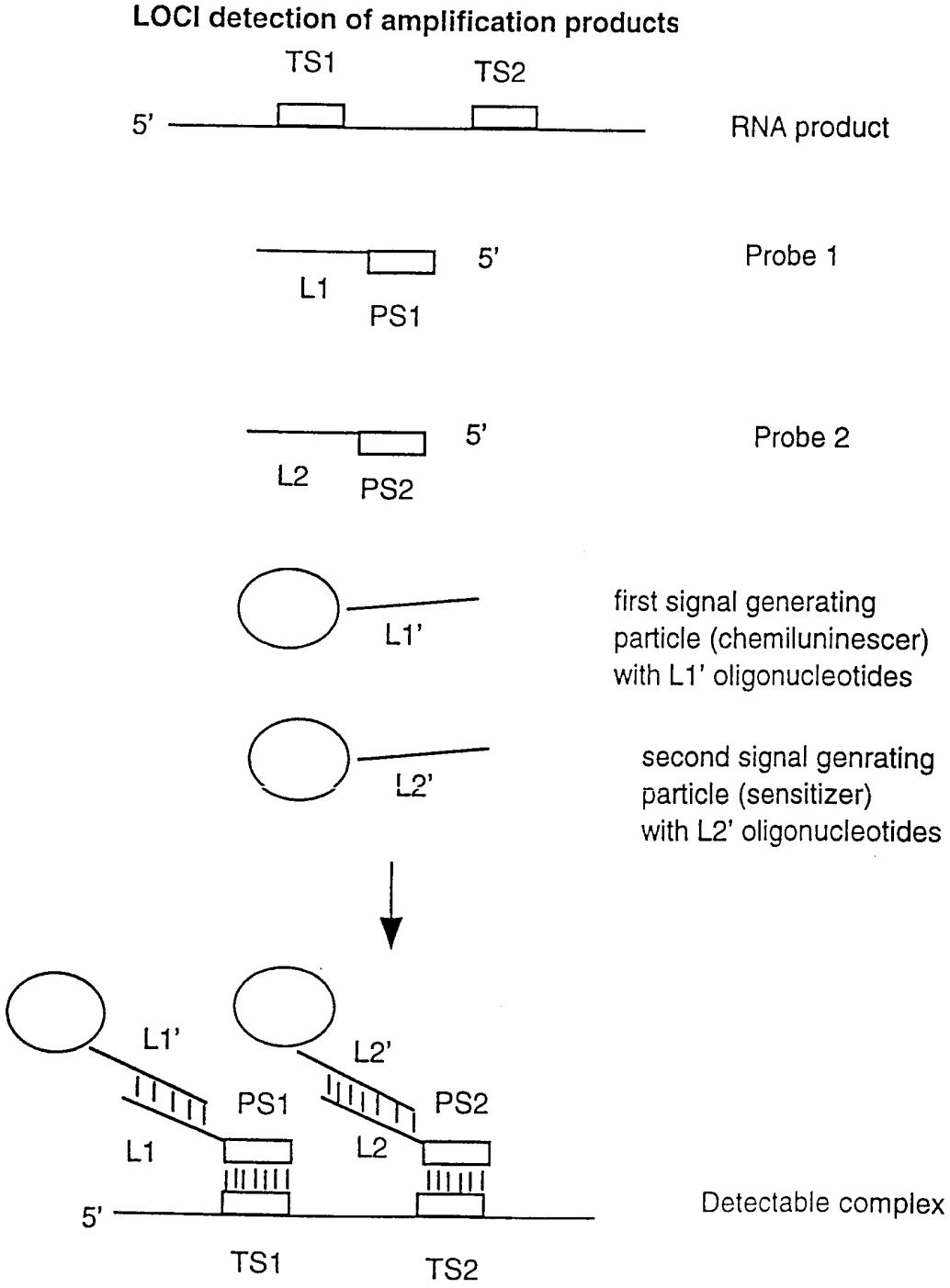

Biot.
t1'
t1
t2
t2'
H
Dig
t1'
t1
Dig
Bio
t2
t2'
Complete strand exchange
Biot.
t1'
t1
M
t2
t2'
H
Dig
t1'
t1'
Biot.
M
H
Dig
t2
t2'
Signal generating cruciform structure

FIG. 7


LOCI detection of amplification products
TS1
TS2
5'
RNA product
5'
L1
PS1
Probe 1
5'
L2
PS2
Probe 2
L1'
first signal generating particle (chemiluninescer) with L1' oligonucleotides
L2'
second signal genrating particle (sensitizer) with L2' oligonucleotides
L1'
L2'
PS1
PS2
L1
L2
5'
TS1
TS2
Detectable complex

SEQUENCE LISTING

<110> Dade Behring Inc.

<120> Homogenous Isothermal Amplification and Detection of Nucleic Acids Using a Template Switch Oligonucleotide

<130> BEH-7418 PCT

<140> PCT/US00/
<141> 2000-05-16

<150> 09/313,240
<151> 1999-05-17

<160> 10

<170> FastSEQ for Windows Version 3.0

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<213> Artifical Sequence

<220>
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<213> Artifical Sequence

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<213> Artifical Sequence

<220>
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