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IDT Exhibit 1020.8277

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IDT Exhibit 1020.8279

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IDT Exhibit 1020.8280

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IDT Exhibit 1020.8281

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IDT Exhibit 1020.8283

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IDT Exhibit 1020.8285

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IDT Exhibit 1020.8286

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IDT Exhibit 1020.8287

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IDT Exhibit 1020.8288

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IDT Exhibit 1020.8289

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IDT Exhibit 1020.8292

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IDT Exhibit 1020.8293

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IDT Exhibit 1020.8296

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IDT Exhibit 1020.8297

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IDT Exhibit 1020.8302

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Espacenet

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METHODS AND COMPOSITIONS FOR AMPLIFICATION OF RNA SEQUENCES

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Abstract of WO02072773 (A2)

The invention provides methods for linear and exponential amplification of RNA. They are particularly suitable for amplifying a plurality of RNA species in a sample. The methods are based on hybridization of polynucleotide comprising a propromoter sequence to a primer extension product to generate an intermediate polynucleotide capable of driving transcription, whereby multiple copies of RNA products comprising sequences complementary to an RNA sequence of interest are generated. The methods are useful for preparation of nucleic acid libraries and substrates for analysis of gene expression of cells in biological samples. The invention also provides compositions and kits for practicing the amplification methods, as well as methods which use the amplification products.

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(54) Title: METHODS AND COMPOSITIONS FOR AMPLIFICATION OF RNA SEQUENCES

(57) Abstract: The invention provides methods for linear and exponential amplification of RNA. They are particularly suitable for amplifying a plurality of RNA species in a sample. The methods are based on hybridization of polynucleotide comprising a propromoter sequence to a primer extension product to generate an intermediate polynucleotide capable of driving transcription, whereby multiple copies of RNA products comprising sequences complementary to an RNA sequence of interest are generated. The methods are useful for preparation of nucleic acid libraries and substrates for analysis of gene expression of cells in biological samples. The invention also provides compositions and kits for practicing the amplification methods, as well as methods which use the amplification products.

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METHODS AND COMPOSITIONS FOR AMPLIFICATION OF RNA SEQUENCES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the priority benefit of the provisional patent application U.S. Serial No. 60/274,236, filed March 9, 2001, which is incorporated by reference in its entirety.

TECHNICAL FIELD

The invention relates to the field of polynucleotide amplification. More particularly, the invention provides methods, compositions and kits for amplifying (i.e., making multiple copies) RNA sequences of interest which employ a polynucleotide comprising a propromoter and RNA transcription.

BACKGROUND ART

The ability to amplify ribonucleic acid (RNA) is an important aspect of efforts to elucidate biological processes. To date, RNA (generally, mRNA) amplification is most commonly performed using the reverse transcriptase-polymerase chain reaction (RT-PCR) method and variations thereof. These methods are based on replication of RNA by reverse transcriptase to form single stranded DNA complementary to the RNA (cDNA), which is followed by polymerase chain reaction (PCR) amplification to produce multiple copies of double stranded DNA. Although these methods are most commonly used, they have some significant drawbacks: a) the reactions require thermocycling; b) the products are double stranded, thus rendering them less accessible to binding to probes; c) the reactions are prone to contamination with products of prior amplification, thus requiring strict containment of reaction mixtures; and d) the exponential nature of amplification of these methods renders them prone to generate pools of products which do not truly reflect the representation of the

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various RNA sequences in the input total RNA sample, due to unequal efficiency of amplification of different sequences, and the nature of exponential amplification which is based on replication of amplification products rather than on continued replication of the input target RNAs.

The total cellular mRNA represents gene expression activity at a defined time. Gene expression is affected by cell cycle progression, developmental regulation, response to internal and external stimuli and the like. The profile of expressed genes for any cell type in an organism reflects normal or disease states, response to various stimuli, developmental stages, cell differentiation, and the like.

Various methods for the analysis of gene expression have be developed in recent years. See, for example, U.S. Pat. Nos. 5,744,308; 6,143,495; 5,824,517; 5,829,547; 5,888,779; 5,545,522; 5,716,785; 5,409,818; EP 0971039A2; EP0878553A2. These include quantification of specific mRNAs, and the simultaneous quantification of a large number of mRNAs, as well as the detection and quantification of patterns of expression of known and unknown genes. The analysis of gene expression profiles is currently one of the most powerful tools in the study of cellular differentiation and cellular development, and in the investigation of normal and disease states of various organisms, in particular in human. This analysis is crucial for gene discovery, molecular medicine and drug discovery processes.

Essential for gene expression profiling is the ability to randomly amplify the total cellular mRNAs prepared from any cell or tissue. Although analysis of non-amplified mRNA is feasible, a significant amount of starting mRNA would be required. However, the total amount of sample mRNA that is available is frequently limited by the amount of biological sample from which it is derived. Biological samples are often limited in amount and precious. Moreover, the amount of the various mRNA species is not equal; some species are more abundant than others, and these are more likely and easier, to analyze. The ability to amplify mRNA sequences enables the analysis of less abundant, rare mRNA species. The ability to analyze small samples, by means of nucleic acid amplification, is also advantageous for design parameters of large scale screening

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of effector molecule libraries, for which reduction in sample volume is a major concern both for the ability to perform very large scale screening or ultra high throughput screening, and in view of the limiting amounts of library components.

Therefore, there is a need for improved RNA amplification methods that overcome drawbacks in existing methods. The invention provided herein fulfills this need and provides additional benefits.

All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

DISCLOSURE OF THE INVENTION

The invention provides methods, compositions, and kits for polynucleotide, specifically ribonucleic acid, amplification, as well as applications of the amplification methods.

In one aspect, the invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising the steps of: (a) extending a first primer hybridized to a target RNA with an RNA-dependent DNA polymerase, whereby a complex comprising a first primer extension product and the target RNA is produced; (b) cleaving RNA in the complex of step (b) with an enzyme that cleaves RNA from an RNA/DNA hybrid; (c) extending a second primer hybridized to the first primer extension product with a DNA-dependent DNA polymerase, whereby a complex comprising the first primer extension product and a second primer extension product is produced; (d) denaturing the complex of step (c); and (e) hybridizing to the second primer extension product a propromoter polynucleotide comprising a propromoter and a region which hybridizes to the second primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the target RNA; whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

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In one aspect, the invention provides methods of generating multiple copies of (amplifying) the complementary sequence of an RNA sequence of interest, said method comprising the steps of: (a) hybridizing a first primer to a target ribonucleic acid; (b) extending the first primer with an RNA-dependent DNA polymerase, whereby a complex comprising a first primer extension product and the target ribonucleic acid is produced; (c) cleaving ribonucleic acid in the complex of step (b) with an enzyme that cleaves RNA from an RNA/DNA hybrid; (d) hybridizing a second primer to the first primer extension product; (e) extending the second primer with a DNA-dependent DNA polymerase, whereby a complex comprising the first primer extension product and a second primer extension product is produced; (f) denaturing the complex of step (e); (g) hybridizing to the second primer extension product a polynucleotide comprising a propromoter and a region which hybridizes to the second primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the target ribonucleic acid, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated. In some embodiments, the polynucleotide comprising a propromoter is a propromoter template oligonucleotide (PTO). In some embodiments, the invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest, said methods comprising the steps of: (a) combining: a single stranded second primer extension product resulting from step (f) of the aspect of the invention described above; a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded second primer extension product; and an RNA polymerase; and (b) incubating the mixture of step (a) under conditions (which includes necessary substrates and buffer conditions) that permit propromoter polynucleotide hybridization and RNA transcription, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated. It is understood that any combination of these incubation steps, and any single incubation step, to the extent that the incubation is performed as part of any of the methods described herein, fall within the scope of the invention. It is also understood that methods that comprise one or

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more incubation steps do not require a separate combination step, as such combinations are implicit in incubating the reaction mixture(s).

In another aspect, the invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising the steps of: (a) extending a first primer hybridized to a target RNA with an RNA-dependent DNA polymerase, whereby a complex comprising a first primer extension product and the target RNA is produced; (b) cleaving RNA in the complex of step (a) with an enzyme that cleaves RNA from an RNA/DNA hybrid; (c) extending a second primer hybridized to the first primer extension product with a DNA-dependent DNA polymerase, whereby a complex comprising the first primer extension product and a second primer extension product is produced; (d) denaturing the complex of step (c); (e) hybridizing to the second primer extension product a propromoter polynucleotide comprising a propromoter and a region which hybridizes to the second primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the target RNA; (f) extending a third primer hybridized to said RNA transcripts with an RNA-dependent DNA polymerase, whereby a complex comprising a third primer extension product and an RNA transcript is produced; (g) cleaving RNA in the complex of step (f) with an enzyme that cleaves RNA from an RNA/DNA hybrid; (h) hybridizing a propromoter polynucleotide comprising a propromoter and a region which hybridizes to a single stranded third primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the target RNA; (i) optionally repeating steps (f) to (h); whereby multiple copies of the complementary sequence of the RNA sequence of interest are produced.

In another aspect, the invention provides methods of generating multiple copies of (amplifying) the complementary sequence of an RNA sequence of interest, said method comprising the steps of: (a) hybridizing a first primer to a target ribonucleic acid; (b) extending the first primer with an RNA-dependent DNA polymerase, whereby a complex comprising a first primer extension product and the target ribonucleic acid is produced; (c) cleaving ribonucleic acid in the

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complex of step (b) with an enzyme that cleaves RNA from an RNA/DNA hybrid; (d) hybridizing a second primer to the first primer extension product; (e) extending the second primer with a DNA-dependent DNA polymerase, whereby a complex comprising the first primer extension product and a second primer extension product is produced; (f) denaturing the complex of step (e); (g) hybridizing to the second primer extension product a propromoter and a region which hybridizes to the second primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the target ribonucleic acid; (h) hybridizing a third primer to said RNA transcripts; (i) extending the third primer with an RNA-dependent DNA polymerase, whereby a complex comprising a third primer extension product and an RNA transcript is produced; (j) cleaving RNA in the complex of step (i) with an enzyme that cleaves RNA from an RNA/DNA hybrid; (k) hybridizing a propromoter polynucleotide comprising a propromoter and a region which hybridizes to a single stranded third primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the target ribonucleic acid; (l) optionally repeating steps (h) to (k), whereby multiple copies of the complementary sequence of the RNA sequence of interest are produced. In some embodiments, the polynucleotide comprising a propromoter is a propromoter template oligonucleotide (PTO). In some embodiments, the invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest, said methods comprising the steps of: (a) combining: a single stranded second primer extension product resulting from step (f) described above in this paragraph; a third primer comprising a sequence hybridizable to an RNA transcript comprising a sequence complementary to the target RNA; a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded second primer extension product; a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded third primer extension product; an enzyme that cleaves RNA from an RNA/DNA hybrid; and an RNA polymerase; and (b) incubating the mixture of step (a) under conditions (which includes necessary

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substrates and buffers) that permit primer extension, RNA cleavage, propromoter polynucleotide hybridization and RNA transcription, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated. In yet another embodiment, the invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest, said methods comprising the steps of: (a) combining: an RNA transcript from step (g) described above in this paragraph; a third primer comprising a sequence hybridizable to the RNA transcript; a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded third primer extension product; an enzyme that cleaves RNA from an RNA/DNA hybrid; and an RNA polymerase; and (b) incubating the mixture of step (a) under conditions (which includes necessary substrates and buffers) that permit primer extension, RNA cleavage, propromoter polynucleotide hybridization and RNA transcription, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

In still another aspect, the invention provides methods of generating multiple copies of (amplifying) the complementary sequence of an RNA sequence of interest, said method comprising the steps of: (a) combining: a target ribonucleic acid; a first primer comprising a sequence that is hybridizable to the target ribonucleic acid; a second primer comprising a sequence hybridizable to an extension product of the first primer; a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded second primer extension product; an RNA-dependent DNA polymerase; a DNA-dependent DNA polymerase; an RNA polymerase; and an enzyme that cleaves RNA from an RNA/DNA hybrid; and (b) incubating the mixture of step (a) under conditions (which includes necessary substrates and buffer conditions) that permit primer hybridization, primer extension, RNA cleavage, propromoter polynucleotide hybridization, and RNA transcription. In some embodiments, the polynucleotide comprising a propromoter is a propromoter template oligonucleotide (PTO).

In yet another aspect, the invention provides methods of generating multiple copies of (amplifying) the complementary sequence of an RNA sequence of interest, said method comprising the steps of: (a) combining: a target

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ribonucleic acid; a first primer comprising a sequence that is hybridizable to the target ribonucleic acid; a second primer comprising a sequence hybridizable to an extension product of the first primer; a third primer comprising a sequence hybridizable to an RNA transcript comprising a sequence complementary to the target ribonucleic acid; a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded second primer extension product; a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded third primer extension product; an RNA-dependent DNA polymerase; a DNA-dependent DNA polymerase; an RNA polymerase; and an enzyme that cleaves RNA from an RNA/DNA hybrid; and (b) incubating the mixture of step (a) under conditions (which includes necessary substrates and buffer conditions) that permit primer hybridization, primer extension, RNA cleavage, propromoter polynucleotide hybridization, and RNA transcription. In some embodiments, the polynucleotide comprising a propromoter is a propromoter template oligonucleotide (PTO).

In another aspect, the invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising the steps of: (a) extending a first primer hybridized to a target RNA with an RNA-dependent DNA polymerase, whereby a complex comprising a first primer extension product and the target RNA is produced; (b) cleaving RNA in the complex of step (b) with an enzyme that cleaves RNA from an RNA/DNA hybrid; (c) extending a second primer hybridized to the first primer extension product with a DNA-dependent DNA polymerase, whereby a complex comprising the first primer extension product and a second primer extension product is produced; (d) denaturing the complex of step (c); and (e) hybridizing to the second primer extension product a propromoter polynucleotide comprising a propromoter and a region which hybridizes to the second primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the target RNA; whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

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In another aspect, the invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising the steps of: (a) extending a first primer hybridized to a target RNA with an RNA-dependent DNA polymerase, whereby a complex comprising a first primer extension product and the target RNA is produced; (b) cleaving RNA in the complex of step (a) with an enzyme that cleaves RNA from an RNA/DNA hybrid; (c) extending a second primer hybridized to the first primer extension product with a DNA-dependent DNA polymerase, whereby a complex comprising the first primer extension product and a second primer extension product is produced; (d) denaturing the complex of step (c); (e) hybridizing to the second primer extension product a propromoter polynucleotide comprising a propromoter and a region which hybridizes to the second primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the target RNA; (f) extending a third primer hybridized to said RNA transcripts with an RNA-dependent DNA polymerase, whereby a complex comprising a third primer extension product and an RNA transcript is produced; (g) cleaving RNA in the complex of step (f) with an enzyme that cleaves RNA from an RNA/DNA hybrid; (h) hybridizing a propromoter polynucleotide comprising a propromoter and a region which hybridizes to a single stranded third primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the target RNA; (i) optionally repeating steps (f) to (h); whereby multiple copies of the complementary sequence of the RNA sequence of interest are produced.

In another aspect, the invention provides a method of generating multiple copies of the complementary sequence of an RNA sequence of interest comprising incubating a reaction mixture, said reaction mixture comprising: (a) a single stranded second primer extension product resulting from step (d) above; (b) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded second primer extension product; and an RNA polymerase; wherein the incubation is under conditions that permit propromoter

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polynucleotide hybridization and RNA transcription, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

In another aspect, the invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest comprising incubating a reaction mixture, said reaction mixture comprising: (a) a single stranded second primer extension product resulting from step (d) above; (b) a third primer comprising a sequence hybridizable to an RNA transcript comprising a sequence complementary to the target RNA; (c) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded second primer extension product; (d) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded third primer extension product; (e) an enzyme that cleaves RNA from an RNA/DNA hybrid; and (f) an RNA polymerase; wherein the incubation is under conditions that permit primer extension, RNA cleavage, propromoter polynucleotide hybridization and RNA transcription, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

In another aspect, the invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising incubating a reaction mixture, said reaction mixture comprising: (a) an RNA transcript from step (e) above, (b) a third primer comprising a sequence hybridizable to the RNA transcript; (c) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded third primer extension product; (d) an enzyme that cleaves RNA from an RNA/DNA hybrid; and (e) an RNA polymerase; wherein the incubation is under conditions that permit primer extension, RNA cleavage, propromoter polynucleotide hybridization and RNA transcription, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

In another aspect, the invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising incubating a reaction mixture, said reaction mixture comprising: (a) a target RNA; (b) a first primer comprising a sequence that is hybridizable to the target RNA; (c) a second primer comprising a sequence

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hybridizable to an extension product of the first primer; (d) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded second primer extension product; (e) an RNA-dependent DNA polymerase; (f) a DNA-dependent DNA polymerase; (g) an RNA polymerase; and (h) an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage, propromoter polynucleotide hybridization, and RNA transcription, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

In another aspect, invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising incubating a reaction mixture, said reaction mixture comprising: (a) a target RNA; (b) a first primer comprising a sequence that is hybridizable to the target RNA; (c) a second primer comprising a sequence hybridizable to an extension product of the first primer; (d) a third primer comprising a sequence hybridizable to an RNA transcript comprising a sequence complementary to the target RNA; (e) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded second primer extension product; (f) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded third primer extension product; (g) an RNA-dependent DNA polymerase;(h) a DNA-dependent DNA polymerase; (i) an RNA polymerase; and (j) an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is conditions that permit primer hybridization, primer extension, RNA cleavage, propromoter polynucleotide hybridization, and RNA transcription, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

In another aspect, the invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising: (a) hybridizing a composite primer to a single stranded second primer extension product resulting from step (d) above, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (b) extending the composite primer with a DNA-dependent DNA polymerase, whereby a

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complex comprising a primer extension product and the second primer extension product is formed; (c) cleaving RNA in the complex of step (b) with an enzyme that cleaves RNA from an RNA/DNA hybrid, such that another composite primer hybridizes to the second primer extension product and repeats primer extension by strand displacement, whereby multiple copies of the complement of the RNA sequence of interest are produced.

In another aspect, the invention provides methods of generating multiple copies of a polynucleotide sequence complementary to an RNA sequence of interest, said method comprising the steps of: (a) extending a composite primer hybridized to a second primer extension product, wherein said primer extension product comprises a complement of a first primer extension product generated by extension of a first primer hybridized to template RNA by any of the methods described herein; whereby said first primer extension product is displaced, and whereby multiple copies of a polynucleotide sequence complementary to the RNA sequence of interest are generated.

In another aspect, the invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising incubating a reaction mixture, said reaction mixture comprising: (a) a single stranded second primer extension product resulting from step (d) above; (b) a composite primer which is hybridizable to the single stranded second primer extension product, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (c) DNA-dependent DNA polymerase; (d) an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is made under conditions that permit composite primer primer hybridization, RNA cleavage, and displacement of the primer extension product from the complex of step (a) described above when its RNA is cleaved and a composite primer binds to the primer extension product in the complex, whereby multiple copies of the complement of the RNA sequence of interest are produced.

In another aspect, the invention provides methods of generating multiple copies of an RNA sequence of interest, said method comprising the steps of: (a) extending a composite primer hybridized to a second primer extension product, wherein said primer extension product comprises a complement of a first primer

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extension product generated by extension of a first primer hybridized to template RNA by any of the methods described herein; whereby said first primer extension product is displaced, (b) hybridizing the displaced first primer extension product with a polynucleotide comprising a propromoter and a region which is hybridizable to the displaced first primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the displaced primer extension products, whereby multiple copies of the RNA sequence of interest are generated.

As is clear to one skilled in the art, reference to production of copies of an RNA or DNA sequence of interest or copies of a polynucleotide sequence complementary to an RNA or DNA sequence of interest refers to products that may contain, comprise or consist of such sequences. As is evident to one skilled in the art, aspects that refer to combining and incubating the resultant mixture also encompasses method embodiments which comprise incubating the various mixtures (in various combinations and/or subcombinations) so that the desired products are formed. It is understood that any combination of these incubation steps, and any single incubation step, to the extent that the incubation is performed as part of any of the methods described herein, fall within the scope of the invention. It is also understood that methods that comprise one or more incubation steps do not require a separate combination step, as such combinations are implicit in incubating the reaction mixture(s)

Various embodiments of the primers are used in the methods of the invention. For example, in some embodiments, the first primer comprises a 5' portion that is not hybridizable (under a given set of conditions) to a target ribonucleic acid. In some of these embodiments, the 5' portion comprises a sequence the complement of which is hybridizable by a propromoter polynucleotide under a given set of conditions. In one example, the presence of said 5' portion in the first primer results in generation of a second primer extension product that is hybridizable (under a given set of conditions) by a propromoter polynucleotide. In another example, the presence of said 5' portion in the first primer results in generation of a third primer extension product that is

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hybridizable (under a given set of conditions) by a propromoter polynucleotide. In some embodiments wherein a target RNA is mRNA, the first primer may comprise a poly-T sequence. In other embodiments, the second primer and the third primer are the same. In still another embodiment, the second primer and the third primer are different. In yet another embodiment, the second primer and the third primer hybridize to different complementary sequences. In some embodiments, the second and/or third primer comprises a sequence (for example, a 3' sequence) that is a random sequence. In yet other embodiments, the second and/or third primer is a random primer. In yet other embodiments, the third primer is a composite primer. In some embodiments, the RNA portion of a composite primer is 5' with respect to the 3' DNA portion. In still other embodiments, the 5' RNA portion is adjacent to the 3' DNA portion. In other embodiments, the composite primer comprises the sequence of a first primer.

The enzymes which may be used in the methods and compositions are described herein. For example, the enzyme that cleaves RNA may be an RNaseH, and the RNA-dependent DNA polymerase may be reverse transcriptase. The RNA-dependent DNA polymerase may comprise an RNase H enzyme activity. Similarly, a DNA polymerase may comprise both RNA-dependent and DNA-dependent DNA polymerase enzyme activities. A DNA-dependent DNA polymerase and an enzyme that cleaves RNA may also be the same enzyme. A DNA-dependent DNA polymerase, an RNA-dependent DNA polymerase, and the enzyme that cleaves RNA can also be the same enzyme.

In some embodiments, methods of the invention are used to generate labeled polynucleotide products (generally DNA or RNA products). In some embodiments of methods for generating labeled DNA products, at least one type of dNTP used is a labeled dNTP. In some embodiments of methods for generating labeled RNA products, at least one type of rNTP used is a labeled rNTP. In other embodiments of methods for generating labeled DNA products, a labeled composite primer is used.

In some embodiments, the methods of the invention employ a propromoter polynucleotide (for example, a PTO) that comprises a region at the 3' end which hybridizes to the second or third primer extension products, whereby DNA

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polymerase extension of the extension products produces a double stranded promoter from which transcription occurs.

The methods are applicable to amplifying any RNA target, including, for example, mRNA and ribosomal RNA. One or more steps may be combined and/or performed sequentially (often in any order, as long as the requisite product(s) are able to be formed). It is also evident, and is described herein, that the invention encompasses methods in which the initial, or first, step is any of the steps described herein. For example, the methods of the invention do not require that the first step be production of the first primer extension product from the RNA template. Methods of the invention encompass embodiments in which later, "downstream" steps are an initial step.

The invention also provides methods which employ (usually, analyze) the products of the amplification methods of the invention, such as sequencing, detection of sequence alteration(s) (e.g., genotyping or nucleic acid mutation detection); determining presence or absence of a sequence of interest; gene expression profiling; subtractive hybridization; preparation of a subtractive hybridization probe; differential amplification; preparation of libraries (including cDNA and differential expression libraries); preparation of an immobilized nucleic acid (which can be a nucleic acid immobilized on a microarray), and characterizing (including detecting and/or quantifying) amplified nucleic acid products generated by the methods of the invention.

In one aspect, the invention provides methods of sequencing an RNA sequence of interest, said method comprising (a) amplifying a target ribonucleic acid containing the sequence of interest by the methods described herein in the presence of a mixture of rNTPs and rNTP analogs such that transcription is terminated upon incorporation of an rNTP analog; and (b) analyzing the amplification products to determine sequence.

In another aspect, the invention provides methods of sequencing an RNA sequence of interest, said method comprising (a) amplifying a target ribonucleic acid containing the sequence of interest by the methods described herein, wherein RNA transcripts generated from the second primer extension product are

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amplified in the presence of a mixture of rNTPs and rNTP analogs such that transcription is terminated upon incorporation of an rNTP analog; and (b) analyzing the amplification products to determine sequence.

In some aspects, the invention provides methods of sequencing an RNA sequence of interest, said methods comprising amplifying a target RNA containing the sequence of interest by the amplification methods of the invention in the presence of a mixture of dNTPs and dNTP analogs (which may be labeled or unlabeled), such that primer extension is terminated upon incorporation of a dNTP analog which may be labeled or unlabeled, and analyzing the amplification products to determine sequence.

In some aspects, the invention provides methods of detecting a mutation (or, in some aspects, characterizing a sequence) in a target ribonucleic acid, comprising (a) amplifying the target ribonucleic acid by a method described herein; and (b) analyzing the amplification products of the method for single stranded conformation, wherein a difference in conformation as compared to a reference single stranded polynucleotide indicates a mutation in the target ribonucleic acid. In other embodiments, the invention provides methods of detecting a mutation (or, in some aspects, characterizing a sequence) in a target ribonucleic acid comprising analyzing amplification products of any of the methods described herein for single stranded conformation, wherein a difference in conformation as compared to a reference single stranded polynucleotide indicates a mutation in the target ribonucleic acid (or, in some aspects, characterizes the target sequence).

In another aspect, the invention provides methods of producing a nucleic acid immobilized to a substrate (which includes methods of producing a microarray), comprising (a) amplifying a target RNA by any of the methods described herein; and (b) immobilizing the amplification products on a substrate. The amplification products can be labeled or unlabeled. In other aspects, the invention provides methods of producing a microarray, comprising (a) amplifying a target RNA by an amplification method described herein; and (b) immobilizing the amplification products on a substrate (which can be solid or semi-solid). In some embodiments, microarrays are produced by immobilizing amplification

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products onto a substrate to make a microarray of amplification products. In other embodiments, microarrays are produced by immobilizing amplification products by any of the methods described herein onto a solid substrate to make a microarray of amplification products. The microarray can comprise at least one amplification product immobilized on a solid or semi-solid substrate fabricated from a material selected from the group consisting of paper, glass, ceramic, plastic, polypropylene, nylon, polyacrylamide, nitrocellulose, silicon an other metals, and optical fiber. An amplification product can be immobilized on the solid or semi-solid substrate in a two-dimensional configuration or a three-dimensional configuration comprising pins, rods, fibers, tapes, threads, beads, particles, microtiter wells, capillaries, and cylinders.

Any of the methods of the invention can be used to generate polynucleotide (generally, RNA or DNA) products that are suitable for characterization of an RNA sequence of interest in a sample. In one embodiment, the invention provides methods for characterizing (for example, detecting and/or quantifying and/or determining presence or absence of) an RNA sequence of interest comprising: (a) amplifying a target RNA by any of the methods described herein; and (b) analyzing the amplification products. Step (b) of analyzing the amplification products can be performed by any method known in the art or described herein, for example by detecting and/or quantifying and/or determining present or absence of amplification products that are hybridized to a probe. These amplification products may or may not be labeled. Any of the methods of the invention can be used to generate polynucleotide (generally, RNA or DNA) products that are labeled by incorporating labeled nucleotides into appropriate step(s) of the methods. These labeled products are particularly suitable for quantification and/or identification and/or determining presence or absence of by methods known in the art, which include the use of arrays such as cDNA microarrays and oligonucleotide arrays. In one aspect, the invention provides a method of characterizing an RNA sequence of interest, comprising (a) amplifying a target RNA by a method described herein to generate labeled products; and (b) analyzing the labeled products. In some embodiments, the step of analyzing RNA products comprises determining amount of said products, whereby the amount of

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the RNA sequence of interest present in a sample is quantified. The polynucleotide products can be analyzed by, for example, contacting them with at least one probe. In some embodiments, the at least one probe is provided as a microarray. The microarray can comprise at least one probe immobilized on a solid or semi-solid substrate fabricated from a material selected from the group consisting of paper, glass, plastic, polypropylene, nylon, polyacrylamide, nitrocellulose, silicon, and optical fiber. A probe can be immobilized on the solid or semi-solid substrate in a two-dimensional configuration or a three-dimensional configuration comprising pins, rods, fibers, tapes, threads, beads, particles, microtiter wells, capillaries, and cylinders.

In another aspect, the invention provides methods of determining gene expression profile in a sample, the methods comprising: (a) amplifying at least one RNA sequence of interest in the sample using any of the methods described herein; and (b) determining amount of amplification products of each RNA sequence of interest, wherein each said amount is indicative of amount of each RNA sequence of interest in the sample, whereby the gene expression profile of the sample is determined.

In another aspect, the invention provides methods of preparing a subtractive hybridization probe, said methods comprising generating multiple single stranded polynucleotide, preferably DNA, copies of the complement of at least one RNA sequences of interest from a first RNA population using any of the methods described herein.

In another aspect, the invention provides methods of performing subtractive hybridization, said methods comprising: (a) generating multiple copies of the complement of at least one RNA sequence of interest from a first RNA population using any of the methods described herein; and (b) hybridizing the multiple copies to a second mRNA population, whereby a subpopulation of the second mRNA population forms a complex with a copy of the complement of at least one RNA sequence of interest. In embodiments in which DNA copies are generated, the methods further comprise: (c) cleaving RNA in the complex of step (b) with an enzyme that cleaves RNA from an RNA/DNA hybrid; and (d) amplifying an unhybridized subpopulation of the second mRNA population (using

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any method, including the methods described herein), whereby multiple copies of single stranded DNA complementary to the unhybridized subpopulation of the second mRNA population are generated.

In another aspect, the invention provides methods for differential amplification, the methods comprising: (a) generating multiple nucleic acid (generally DNA) copies of the complement of at least one RNA sequence of interest from a first RNA population using any of the methods described herein; (b) hybridizing the multiple copies to a second mRNA population, whereby a subpopulation of the second mRNA population forms a complex with a DNA copy; (c) cleaving RNA in the complex of step (b) with an enzyme that cleaves RNA from an RNA/DNA hybrid; and (d) amplifying an unhybridized subpopulation of the second mRNA population using any method, including those described herein, whereby multiple copies of single stranded DNA complementary to the unhybridized subpopulation of the second mRNA population are generated. These methods encompass steps (b), (c) and (d) if the copies used in the subtractive hybridization are generated using any of the methods described herein.

In another aspect, the invention provides methods for making a library, said method comprising preparing a subtractive hybridization probe as described herein, or differential amplification as described herein. Any of these applications can use any of the amplification methods (including various components and various embodiments of any of the components) as described herein.

The invention also provides compositions, kits, complexes, reaction mixtures and systems comprising various components (and various combinations of the components) used in the amplification methods described herein. The compositions may be any component(s), reaction mixture and/or intermediate described herein, as well as any combination thereof.

In some embodiments, the invention provides a composition comprising: (a) a first primer (which can be a random primer); (b) a second primer (which can be a random primer); and (c) a propromoter polynucleotide (which in some embodiments is a PTO). In some embodiments, these compositions may further comprise: (d) a third primer (which can be a random primer). In some of these

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embodiments, the first primer comprises a sequence that is not hybridizable to a target RNA. In some of these embodiments, the second primer comprises a sequence that is not hybridizable to a first primer extension product. In some embodiments, the third primer comprises a sequence that is not hybridizable to an RNA transcript. In some embodiments, the propromoter polynucleotide ((c), above) is capable of hybridizing to the complement of the 5' portion of the first primer.

The invention also provides compositions comprising a propromoter polynucleotide (such as a PTO) capable of hybridizing to a 3' portion of a second primer extension that is complement of a 5' portion of a first primer used to create first primer extension product.

The invention also provides compositions comprising (a) a first primer; (b) a second primer (which can be a random primer); and (c) a composite primer, wherein the composite primer comprises a 5' RNA portion and a DNA portion. In some embodiments, the invention provides a composition comprising: (a) a first primer (which can be a random primer) hybridizable to target RNA; (b) a second primer (which can be a random primer); and (c) a composite primer hybridizable to a second primer extension product. In some embodiments, the composition further comprises one or more of the following: DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, and an agent (generally an enzyme) that cleaves RNA from an RNA/DNA heteroduplex.

The invention also provides compositions comprising the amplification products described herein. Accordingly, the invention provides a population of anti-sense RNA molecules which are copies of a target sequence, which are produced by any of the methods described herein. The invention also provides a population of anti-sense polynucleotides (generally DNA) molecules, which are produced by any of the methods described herein.

In another aspect, the invention provides compositions comprising any of the complexes (which are generally considered as intermediates with respect to the final amplification products) described herein (see also the figures for schematic depictions of examples of these various complexes). For example, the invention provides compositions comprising a complex of (a) a first primer

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extension product; and (b) a target RNA strand. In yet another aspect, the invention provides compositions comprising a complex of: (a) a first primer extension product; and (b) a second primer extension product. In another example, the invention provides compositions comprising a complex of (a) a second primer extension product; and (b) a propromoter polynucleotide (which can be a PTO). In some embodiments, the propromoter polynucleotide hybridizes to a sequence in the second primer extension product comprising the complement of the 5' portion of a first prime, wherein the first primer is extended to form the first primer extension product. In yet another example, the invention provides compositions comprising a complex of (a) a third primer extension product; and (b) a propromoter polynucleotide (which can be a PTO). In yet another example, the invention provides compositions comprising a complex of (a) a second primer extension product, generated by denaturation of a hybridized first and second primer extension product; and (b) a composite primer hybridizable to the second primer extension product.

In another aspect, the invention includes any one or more products (including intermediates) and compositions comprising the products (including intermediates) produced by any aspect of the methods of the invention. The products include libraries and any other population produced, which are generally based on the nature of the primer(s) used in the methods described herein.

In another aspect, the invention provides reaction mixtures (or compositions comprising reaction mixtures) which contain various combinations of components described herein. For example, the invention provides reaction mixtures comprising (a) a target RNA; (b) a first primer; (c) a second primer (which can be a random primer); (d) an RNA polymerase; and (e) a DNA polymerase. The reaction mixture could also further comprise an enzyme which cleaves RNA from an RNA/DNA hybrid, such as RNase H. A reaction mixture of the invention can also comprise a propromoter polynucleotide (which in some embodiments is a PTO).

In another aspect, the invention provides reaction mixtures comprising (a) a target RNA; (b) a first primer; (c) a second primer (which can be a random primer); (d) an RNA polymerase; (e) a DNA polymerase; and (f) a composite

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primer. The reaction mixture could also further comprise an enzyme which cleaves RNA from an RNA/DNA hybrid, such as RNase H.

In another aspect, the invention provides kits for conducting the methods described herein. These kits, in suitable packaging and generally (but not necessarily) containing suitable instructions for performing any of the methods of the invention described herein, including sequencing, detection of sequence alteration(s) (e.g., genotyping or nucleic acid mutation detection); determining presence or absence of a sequence of interest; gene expression profiling; subtractive hybridization; preparation of a subtractive hybridization probe; differential amplification; preparation of libraries (including cDNA and differential expression libraries); preparation of an immobilized nucleic acid (which can be a nucleic acid immobilized on a microarray), and characterizing (including detecting and/or quantifying and/or determining presence or absence of) amplified nucleic acid products generated by the methods of the invention. The kits further comprise one or more components used in the methods of the invention. For example, the invention provides kits that comprise a first primer that comprises a sequence the complement of which is hybridizable by a propromoter polynucleotide, and instructions for using the primer to amplify RNA. The invention also provides kits that further comprise a second primer and/or a third primer, and optionally instructions for using the primers to amplify RNA. The kits can contain further components, such as any of (a) a propromoter polynucleotide (such as a PTO); and (b) any of the enzymes described herein, such as an enzyme which cleaves RNA from an RNA/DNA hybrid (for example, RNaseH), DNA polymerase (RNA-dependent or DNA-dependent) and RNA polymerase. In another example, a kit can comprise (a) a composite primer; and (b) instructions for using the composite primer to amplify target RNA using the methods of the invention provided herein. The kit can comprise further components, including any of the enzymes described herein, such as an enzyme which cleaves RNA from an RNA/DNA hybrid (for example, RNaseH), and DNA polymerase (RNA-dependent or DNA-dependent). In another example, a kit comprises a first primer that comprises a sequence the complement of which is hybridizable by a propromoter polynucleotide, and instructions for using the

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primer to amplify RNA using any of the methods described herein. In another embodiment, the kit further comprises a second primer.

In another aspect, the invention provides systems for effecting the amplification methods described herein. For example, the invention provides systems for amplifying a target ribonucleic acid, comprising: (a) a first primer; (b) a second primer (which can be a random primer); (c) an RNA-dependent DNA polymerase; (d) a DNA-dependent DNA polymerase; (e) a propromoter polynucleotide (such as a PTO); and (f) an enzyme which cleaves RNA from an RNA/DNA hybrid (such as RNaseH). The systems may also comprise: (g) a third primer (which can be a random primer). As described herein, systems of the invention generally comprise one or more apparatuses appropriate for carrying out methods of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a diagrammatic representation of a linear RNA amplification process. Figure 1 shows amplification of a target RNA using a polynucleotide comprising a propromoter to produces multiple copies of RNA transcripts complementary to the target RNA.

FIGURES 2A-B show further amplification of the RNA transcripts from the process of Figure 1 to generate more RNA transcripts complementary to the target RNA.

FIGURES 3A-B show a diagrammatic representation of a linear RNA amplification process using a third primer that is a composite primer to generate single stranded DNA strands complementary to the target RNA.

MODES FOR CARRYING OUT THE INVENTION

The invention provides methods, compositions and kits for amplifying polynucleotide sequences, specifically ribonucleic acid (RNA) sequences. The methods provide for amplification of a single RNA species or pool of RNA species. The methods can achieve exponential amplification, which would be

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particularly useful for amplification of very low amounts of RNA sequences in a biological sample. The methods are suitable for, for example, generation of libraries, including cDNA libraries. They generate single stranded RNA or, in some embodiments, single stranded DNA products, which are readily suitable for multiplex analysis by microarray technologies, as well as electrophoresis-based technologies such as differential display. The methods are amenable to automation and do not require thermal cycling. The methods generally comprise hybridizing a polynucleotide comprising a propromoter sequence to a primer extension product to generate an intermediate product capable of driving transcription, whereby RNA transcripts comprising sequences complementary to an RNA sequence of interest are produced. In another aspect, the methods comprise isothermal linear amplification of DNA copies complementary to the RNA sequence of interest using a denaturation step, a composite primer and strand displacement. See Kurn, U.S. Patent No. 6,251,639 B1.

The methods of the invention are directed to the amplification of one or more species of RNA, such as a pool of RNA sequences, and is most particularly suitable for the amplification of all RNA (such as mRNA) sequences in a preparation of total RNA from a biological sample. Thus, one of the major advantages of the methods of the invention is the ability to amplify an entire pool of sequences, which is essential for the ability to analyze the gene expression profile in cells, such as the cells in a biological of interest sample. The methods of the invention have the potential of amplifying a multiplicity, more preferably a large multiplicity, and most preferably all RNA (such as mRNA) sequences in a sample.

Insofar as many mRNAs have a unique polyA 3'-end, the amplification initiated from the 3'-end sequence of mRNAs is most common for preparation of cDNA libraries and subsequent sequence analysis for determination of gene expression profiling or other applications. The methods of the invention are similarly suited for preparation of libraries of amplified 3'-portions of mRNAs. The sequence of the first primer used in the methods of invention can be designed to be complementary to a multiplicity, or all, of the mRNA species in the sample by using random sequences, according to methods known in the art.

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Various methods for mRNA amplification have been described. U.S. Pat. Nos. ,744,308; 6,143,495; EP 0971039A2; EP0878553A2. Most of these methods are transcription based, wherein a promoter for RNA polymerase is incorporated into a double stranded cDNA by a primer comprising a propromoter sequence at the 5'-end which hybridizes to target RNA. These primers can non-specifically bind to template RNA. Insofar as a DNA polymerase has a high affinity for primer hybridized to a template nucleic acid with a free 3' end, i.e. a substrate for primer extension by the polymerase, it is highly probable that a primer comprising a propromoter sequence at the 5' end may non-specifically incorporate the promoter sequence into an amplification product. This results in uncontrolled production of transcription products. The appending of a double stranded promoter by a propromoter polynucleotide, as described herein, provides for increased specificity and control of the transcription-based generation of amplification product.

In one aspect, the invention works as follows: generation of multiple copies of the complementary sequence of an RNA sequence of interest is achieved by using a first primer (which can be a specific or random primer) that comprises a sequence (generally, in its 5' portion) the complement of which is hybridizable by a polynucleotide comprising a propromoter. In some embodiments, the sequence the complement of which is hybridizable by a polynucleotide comprising a propromoter is a sequence that is hybridizable to a target RNA when the primer is hybridized to the target RNA. In other embodiments, the sequence the complement of which is hybridizable by a polynucleotide comprising a propromoter is a sequence that is not hybridizable to a target RNA when the primer is hybridized to the target RNA (thus forming a tail when the primer is hybridized to a target). The extension of the first primer along a target RNA by an RNA-dependent DNA polymerase results in the generation of an intermediate polynucleotide (first primer extension product) that has at least one defined end (the first primer end). After cleavage of the template RNA from a complex comprising the target RNA and first primer extension product, a second primer (which can be a specific or random primer) is then hybridized to the first DNA strand (first primer extension product) and extended to form a complex of first

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and second primer extension products that at one end comprises a sequence to which a polynucleotide comprising a propromoter is hybridizable. The second primer is any sequence that is hybridized to the first DNA strand such that it is capable of being extended by a DNA polymerase along a first DNA strand to generate a second DNA strand. Thus, in some embodiments, the second primer is an oligonucleotide (that is separately provided). In other embodiments, it is or comprises a sequence of the first DNA strand (generally at the 3' end) that is hybridized to a sequence in the first DNA strand (for example, a hairpin or self-annealed structure). Following denaturation of the complex, a polynucleotide comprising a propromoter is hybridized to the second primer extension product and the 3' end of the primer extension product is extended along the propromoter oligonucleotide (if there is any overhang) to generate a double stranded promoter region. RNA transcription driven by this promoter results in generation of multiple copies of RNA transcripts comprising the complementary sequence of the RNA sequence of interest. In some embodiments involving cyclical amplification (also referred to herein as "exponential" amplification), these RNA transcripts, and optionally RNA transcripts generated in subsequent steps, are hybridized with a third primer (which may or may not be the same as the second primer, and which may be a specific or random primer). The third primer is extended to form a complex comprising the RNA transcript and a third primer extension product (which constitutes a DNA/RNA heteroduplex). Cleavage of the RNA transcript results in a single stranded third primer extension product to which a propromoter polynucleotide hybridizes. If necessary (i.e., if there is an overhang), the third primer extension product is extended along the propromoter polynucleotide to generate a double stranded promoter region. RNA transcription driven by this promoter results in generation of multiple copies of RNA transcripts comprising the complementary sequences of the RNA sequence of interest. Hybridization of third primers to these RNA transcripts initiates a cyclical process leading to further amplification.

Accordingly, the invention provides methods of producing at least one copy of the complementary sequence of an RNA sequence of interest, said method comprising combining and reacting the following: (a) a target RNA

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comprising an RNA sequence of interest; (b) a first primer that hybridizes to the target RNA; (c) a second primer that is hybridizable to an extension product of the first primer; (d) an RNA-dependent DNA polymerase; (e) a DNA-dependent DNA polymerase; (f) an enzyme that cleaves RNA from an RNA/DNA hybrid; (g) a propromoter polynucleotide comprising a propromoter and a region which hybridizes to an extension product of the second primer; (h) deoxyribonucleoside triphosphates or suitable analogs; (i) ribonucleoside triphosphates and suitable analogs; and (j) an RNA polymerase. In embodiments involving cyclical amplification (interchangeably termed "exponential" amplification, herein), the following are also included in the amplification reaction (either at the same time as the components listed above or added separately): (k) optionally a third primer (which may or may not be the same as the second primer) that hybridizes to an RNA transcript comprising sequences complementary to the sequence of the target RNA; and (l) optionally a second polynucleotide comprising a propromoter and a region which hybridizes to a single stranded third primer extension product (this polynucleotide may or may not be the same as the polynucleotide described in (g) above).

As is evident to one skilled in the art, by this disclosure, the reactions described may be simultaneous or sequential, as such, the invention includes these various embodiments and combinations.

In some embodiments, the invention provides methods of producing at least one copy of the complementary sequence of an RNA sequence of interest, said method comprising combining and reacting the following: (a) a single stranded second primer extension product resulting from denaturation of a complex of first and second primer extension products as described herein; (b) a propromoter polynucleotide comprising a propromoter and a region which hybridizes to a second primer extension product; (c) ribonucleoside triphosphates and suitable analogs; and (d) an RNA polymerase. In embodiments involving cyclical amplification ("exponential" amplification), the following may also included in the amplification reaction (either at the same time as the components listed above or added separately): (e) optionally a third primer (which may or may not be the same as the second primer) that hybridizes to an RNA transcript

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comprising sequences complementary to the sequence of the target RNA; and (f) optionally a second polynucleotide comprising a propromoter and a region which hybridizes to a single stranded third primer extension product (this polynucleotide may or may not be the same as the polynucleotide described in (b) above). In some embodiments involving cyclical amplification, said method comprises combining and reacting (under suitable conditions and reagent such that multiple copies of a polynucleotide sequence complementary to an RNA sequence of interest are produced) the following: (a) an RNA transcript generated from the complex of the first propromoter polynucleotide and second primer extension product; (b) a third primer (which may or may not be the same as the second primer) that hybridizes to the RNA transcript; (c) a second polynucleotide comprising a propromoter and a region which hybridizes to a single stranded third primer extension product (this polynucleotide may or may not be the same as the propromoter polynucleotide in the complex from which the RNA transcript in step (a) is generated).

In another aspect, the invention provides a method of generating multiple copies of a polynucleotide sequence complementary to an RNA sequence of interest as follows: generation of multiple copies of the complementary sequence of an RNA sequence of interest is achieved by using a first primer (which can be a specific or random primer) that comprises a sequence in its 5' portion that is not hybridizable to target RNA (thus forming a tail when the primer is hybridized to a target under conditions when the first primer hybridizes to template RNA). The extension of the first primer along a target RNA by an RNA-dependent DNA polymerase results in the generation of an intermediate polynucleotide (first strand cDNA) that has at least one defined end comprising the complement of the first primer sequence. After cleavage of the template RNA from a complex comprising the target RNA and first strand cDNA, a second primer (which can be a specific or random primer) is then hybridized to the first strand cDNA) and extended to form a complex of first and second strand cDNAs that at (at least) one end has a defined end comprising the first primer sequence and the complement of the first primer sequence. The second primer is any sequence that is hybridized to the first DNA strand such that it is capable of being extended by a DNA

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polymerase along a first DNA strand to generate a second DNA strand. Thus, in some embodiments, the second primer is an oligonucleotide (that is separately provided). In other embodiments, it comprises a sequence of the first DNA strand (generally at the 3' end) that is hybridized to a sequence in the first DNA strand (for example, a hairpin or self-annealed structure). In other embodiment, the second primer comprises one or more fragments of the target RNA sequence that remains hybridized to the first primer extension product (after cleavage of the initial complex comprising the target RNA and first primer extension product). The complex of first and second strand cDNAs (wherein the second strand cDNA comprises a 3' end portion that is the complement of the first primer sequence) is then denatured to form single stranded first strand cDNA and second strand cDNA.

The single stranded second strand cDNA is the substrate for isothermal linear amplification using a composite primer and strand displacement as follows: a composite primer comprising a 5'-RNA portion and a DNA portion hybridizes to the 3'-portion of the second strand cDNA, generally to the 3'-portion of the second strand cDNA, and is extended along the second strand cDNA by a DNA polymerase to form a double stranded complex comprising an RNA/DNA hybrid portion at one end of the complex. An enzyme which cleaves RNA from an RNA/DNA hybrid (such as RNase H) cleaves RNA sequence from the hybrid, leaving a sequence on the second strand cDNA available for binding by another composite primer. Another first (composite) strand cDNA is produced by DNA polymerase, which displaces the previously bound cleaved first strand cDNA, resulting in displaced cleaved first strand cDNA. The displaced cleaved first strand cDNA product comprises single stranded DNA complementary to the RNA sequence of interest. Kurn, U.S. Patent No. 6,251,639 B1.

Any of the methods of the invention can be used to generate polynucleotide (generally, RNA or DNA) products that are labeled by incorporating labeled nucleotides into appropriate steps of the methods. These labeled products are particularly suitable for quantification and/or identification by methods known in the art, which include the use of arrays such as cDNA microarrays and oligonucleotide arrays.

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In some embodiments, the invention provides methods of sequencing RNA sequences. For sequencing methods based on methods described herein, the appropriate rNTPs, or analogs thereof, which may be labeled or unlabeled, are used. Accordingly, the invention provides methods of sequencing a target RNA comprising a sequence of interest based on the methods described above, wherein rNTPs and rNTP analogs which are primer elongation terminators, which may be labeled or unlabeled, are used, and the amplification product is analyzed for sequence information, as described below. For sequencing methods based on methods described herein wherein the amplified product is DNA, the appropriate dNTPs, or analogs thereof, which may be labeled or unlabeled, are used.

In other embodiments, the invention provides methods of detecting nucleic acid sequence mutations. In one embodiment, the amplification products are used to detect and/or identify single strand conformation polymorphisms in a target RNA.

The invention provides methods to characterize (for example, detect and/or quantify and/or determine presence or absence of) an RNA sequence of interest by generating polynucleotide (generally RNA or DNA) products using amplification methods of the invention, and analyzing the products by detection/quantification methods such as those based on array technologies or solution phase technologies. Generally, but not necessarily, these amplified products are labeled.

In another aspect, the invention provides a method of characterizing an RNA sequence of interest, comprising (a) amplifying a target RNA by a method described herein to generate labeled products; and (b) analyzing the labeled polynucleotide (generally, RNA or DNA) products. In some embodiments, the step of analyzing products comprises determining amount of said products, whereby the amount of the RNA sequence of interest present in a sample is quantified. The polynucleotide (generally, DNA or RNA) products can be analyzed by, for example, contacting them with at least one probe. In some embodiments, the at least one probe is provided as a microarray. The microarray can comprise at least one probe immobilized on a solid or semi-solid substrate fabricated from a material selected from the group consisting of paper, glass,

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ceramics, plastic, polypropylene, polystyrene, nylon, polyacrylamide, nitrocellulose, silicon, other metals, and optical fiber. A probe can be immobilized on the solid or semi-solid substrate in a two-dimensional configuration or a three-dimensional configuration comprising pins, rods, fibers, tapes, threads, beads, particles, microtiter wells, capillaries, and cylinders.

In another aspect, the invention provides methods of determining gene expression profile in a sample, the methods comprising: (a) amplifying at least one RNA sequence of interest in the sample using any of the methods described herein; and (b) determining amount of amplification products of each RNA sequence of interest, wherein each said amount is indicative of amount of each RNA sequence of interest in the sample, whereby the gene expression profile of the sample is determined.

In another embodiment, the invention provides methods of generating libraries (including cDNA libraries and subtractive hybridization libraries), said methods comprising: amplifying at least one RNA sequences of interest using any of the methods described herein to generate single stranded nucleic acid product, and methods of generating and using subtractive hybridization probes, methods for subtractive hybridization, methods for differential amplification, and methods of generating subtractive hybridization libraries.

Various methods for the detection and quantification of gene expression levels have been developed in recent years. For example, microarrays, in which either defined cDNAs or oligonucleotides are immobilized at discrete locations on, for example, solid or semi-solid substrates, or on defined particles, enables the detection and/or quantification of the expression of a multitude of genes in a given specimen.

Using these previously known methods to detect and/or quantify multiple mRNA species in a sample, which in turn is a measure of gene expression profiling, generally requires direct labeling of cDNA, which requires a large amount of input total RNA, in part because mRNA represents only a small subset of the total RNA pool. Thus, when using total RNA preparations from a given cell or tissue sample, the analysis of gene expression in the sample using methods such as arrays requires a substantial amount of input RNA, which generally

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ranges from 50 to 200 μg. Similarly, 2 to 5 μg of mRNA purified from a total RNA preparation would generally be required for a single analysis of gene expression profiling using array technologies. This is a clear limitation of methods such as those based on array technology, insofar as the number of cells, or size of tissue specimen required is very large, and these cells or tissue specimens are often scarcely available for testing or are too precious. This limitation is especially severe in the study of clinical specimens, where the cells to be studied are rare and/or difficult to cultivate *in vitro*, and in high throughput screening of libraries of effector molecules.

General Techniques

The practice of the invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994).

Primers, oligonucleotides and polynucleotides employed in the invention can be generated using standard techniques known in the art.

Definitions

A "target sequence," "target nucleic acid," or "target RNA," as used herein, is a polynucleotide comprising a sequence of interest, for which amplification is desired. The target sequence may be known or not known, in terms of its actual sequence. In some instances, the terms "target" and "template", and variations thereof, are used interchangeably.

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"Amplification," as used herein, generally refers to the process of producing multiple copies of a desired sequence. "Multiple copies" mean at least 2 copies. A "copy" does not necessarily mean perfect sequence complementarity or identity to the template sequence. For example, copies can include nucleotide analogs such as deoxyinosine, intentional sequence alterations (such as sequence alterations introduced through a primer comprising a sequence that is hybridizable, but not complementary, to the template, or a non-target sequence introduced through a primer), and/or sequence errors that occur during amplification. "Amplifying" a sequence may generally refer to making copies of a sequence or its complement, with the understanding that, as stated above, copying does not necessarily mean perfect sequence complementarity or identity with respect to the template sequence.

"Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, cabamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, ply-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the

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polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping groups moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'--O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, α-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), "(O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

A "labeled dNTP," or "labeled rNTP," as used herein, refers, respectively, to a dNTP or rNTP, or analogs thereof, that is directly or indirectly attached with a label. For example, a "labeled" dNTP or rNTP, may be directly labeled with, for example, a dye and/or a detectable moiety, such as a member of a specific binding pair (such as biotin-avidin). A "labeled" dNTP or rNTP, may also be indirectly labeled by its attachment to, for example, a moiety to which a label is/can be attached. A dNTP or rNTP, may comprise a moiety (for example, an amine group) to which a label may be attached following incorporation of the dNTP or rNTP into an extension product. Useful labels in the present invention include fluorescent dyes (e.g., fluorescein isothiocyanate, Texas red, rhodamine, green fluorescent protein and the like), radioisotopes (e.g., ^{3}H, ^{35}S, ^{32}P, ^{33}P, ^{125}I, or

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^{14}C), enzymes (e.g., LacZ, horseradish peroxidase, alkaline phosphatase,), digoxigenin, and colorimetric labels such as colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads. Various anti-ligands and ligands can be used (as labels themselves or as a means for attaching a label). In the case of a ligand that has a natural anti-ligand, such as biotin, thyroxine and cortisol, the ligand can be used in conjunction with labeled anti-ligands.

The "type" of dNTP or rNTP, as used herein, refers to the particular base of a nucleotide, namely adenine, cytosine, thymine, uridine, or guanine.

"Oligonucleotide," as used herein, generally refers to short, generally single stranded, generally synthetic polynucleotides that are generally, but not necessarily, less than about 200 nucleotides in length. Oligonucleotides in the invention include the first, second, and third primers, and propromoter polynucleotide (such as the PTO). The terms "oligonucleotide" and "polynucleotide" are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides.

A "primer" is generally a nucleotide sequence (i.e. a polynucleotide), generally with a free 3'-OH group, that hybridizes with a template sequence (such as a target RNA, or a primer extension product) and is capable of promoting polymerization of a polynucleotide complementary to the template. A "primer" can be, for example, an oligonucleotide. It can also be, for example, a sequence of the template (such as a primer extension product or a fragment of the template created following RNase cleavage of a template-DNA complex) that is hybridized to a sequence in the template itself (for example, as a hairpin loop), and that is capable of promoting nucleotide polymerization. Thus, a primer can be an exogenous (e.g., added) primer or an endogenous (e.g., template fragment) primer.

A "random primer," as used herein, is a primer that comprises a sequence that is designed not necessarily based on a particular or specific sequence in a sample, but rather is based on a statistical expectation (or an empirical observation) that the sequence of the random primer is hybridizable (under a given set of conditions) to one or more sequences in the sample. The sequence of a random primer (or its complement) may or may not be naturally-occurring, or

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may or may not be present in a pool of sequences in a sample of interest. The amplification of a plurality of RNA species in a single reaction mixture would generally, but not necessarily, employ a multiplicity, preferably a large multiplicity, of random primers. As is well understood in the art, a "random primer" can also refer to a primer that is a member of a population of primers (a plurality of random primers) which collectively are designed to hybridize to a desired and/or a significant number of target sequences. A random primer may hybridize at a plurality of sites on a nucleic acid sequence. The use of random primers provides a method for generating primer extension products complementary to a target polynucleotide which does not require prior knowledge of the exact sequence of the target.

"Protopromoter sequence," and "propromoter sequence," as used herein, refer to a single-stranded DNA sequence region which, in double-stranded form is capable of mediating RNA transcription. In some contexts, "protopromoter sequence," "protopromoter," "propromoter sequence," "propromoter," "promoter sequence," and "promoter" are used interchangeably.

A "propromoter polynucleotide," as used herein, refers to a polynucleotide comprising a propromoter sequence. Example of a propromoter polynucleotide is a propromoter template oligonucleotide (PTO).

"Propromoter template oligonucleotide (PTO)"and "promoter template oligionucleotide (PTO)" as used herein, refer to an oligonucleotide that comprises a propromoter sequence and a portion, generally a 3' portion, that is hybridizable (under a given set of conditions) to the 3' region of a primer extension product. The propromoter sequence and the hybridizable portion may be the same, distinct or overlapping nucleotides of an oligonucleotide.

To "inhibit" is to decrease or reduce an activity, function, and/or amount as compared to a reference.

A "complex" is an assembly of components. A complex may or may not be stable and may be directly or indirectly detected. For example, as is described herein, given certain components of a reaction, and the type of product(s) of the reaction, existence of a complex can be inferred. For purposes of this invention, a complex is generally an intermediate with respect to the final amplification

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product(s). An example of a complex is a nucleic acid duplex comprising a first primer extension product and a second primer extension product.

"Denaturing," or "denaturation of" a complex comprising two polynucleotides (such as a first primer extension product and a second primer extension product) refers to dissociation of two hybridized polynucleotide sequences in the complex. The dissociation may involve a portion or the whole of each polynucleotide. Thus, denaturing or denaturation of a complex comprising two polynucleotides can result in complete dissociation (thus generating two single stranded polynucleotides), or partial dissociation (thus generating a mixture of single stranded and hybridized portions in a previously double stranded region of the complex).

A "portion" or "region," used interchangeably herein, of a polynucleotide or oligonucleotide is a contiguous sequence of 2 or more bases. In other embodiments, a region or portion is at least about any of 3, 5, 10, 15, 20, 25 contiguous nucleotides.

A region, portion, or sequence which is "adjacent" to another sequence directly abuts that region, portion, or sequence.

A "reaction mixture" is an assemblage of components, which, under suitable conditions, react to form a complex (which may be an intermediate) and/or a product(s).

"A", "an" and "the", and the like, unless otherwise indicated include plural forms.

"Comprising" means including.

Conditions that "allow" an event to occur or conditions that are "suitable" for an event to occur, such as hybridization, strand extension, and the like, or "suitable" conditions are conditions that do not prevent such events from occurring. Thus, these conditions permit, enhance, facilitate, and/or are conducive to the event. Such conditions, known in the art and described herein, depend upon, for example, the nature of the nucleotide sequence, temperature, and buffer conditions. These conditions also depend on what event is desired, such as hybridization, cleavage, strand extension or transcription.

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Sequence "mutation," as used herein, refers to any sequence alteration in a sequence of interest in comparison to a reference sequence. A reference sequence can be a wild type sequence or a sequence to which one wishes to compare a sequence of interest. A sequence mutation includes single nucleotide changes, or alterations of more than one nucleotide in a sequence, due to mechanisms such as substitution, deletion or insertion. Single nucleotide polymorphism (SNP) is also a sequence mutation as used herein.

"Single stranded conformation polymorphism," and "SSCP," as used herein, generally refer to the specific conformation of a single stranded nucleic acid as is affected by its specific nucleic acid sequence. Alteration of the sequence of the single stranded polynucleotide, such as single nucleotide substitution, deletions or insertions, result in change, or polymorphism, of the conformation of the single stranded polynucleotide. The conformation of the polynucleotide is generally detectable, identifiable and/or distinguishable using methods known in the art, such as electrophoretic mobility as measured by gel electrophoresis, capillary electrophoresis, and/or susceptibility to endonuclease digestion.

"Microarray" and "array," as used interchangeably herein, comprise a surface with an array, preferably ordered array, of putative binding (e.g., by hybridization) sites for a biochemical sample (target) which often has undetermined characteristics. In a preferred embodiment, a microarray refers to an assembly of distinct polynucleotide or oligonucleotide probes immobilized at defined positions on a substrate. Arrays are formed on substrates fabricated with materials such as paper, glass, ceramic, plastic (e.g., polypropylene, nylon, polystyrene), polyacrylamide, nitrocellulose, silicon or other metals, optical fiber or any other suitable solid or semi-solid support, and configured in a planar (e.g., glass plates, silicon chips) or three-dimensional (e.g., pins, fibers, beads, particles, microtiter wells, capillaries) configuration. Probes forming the arrays may be attached to the substrate by any number of ways including (i) *in situ* synthesis (e.g., high-density oligonucleotide arrays) using photolithographic techniques (see, Fodor et al., *Science* (1991), 251:767-773; Pease et al., *Proc. Natl. Acad. Sci. U.S.A.* (1994), 91:5022-5026; Lockhart et al., *Nature Biotechnology* (1996),

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14:1675; U.S. Pat. Nos. 5,578,832; 5,556,752; and 5,510,270); (ii) spotting/printing at medium to low-density (e.g., cDNA probes) on glass, nylon or nitrocellulose (Schena et al, *Science* (1995), 270:467-470, DeRisi et al, *Nature Genetics* (1996), 14:457-460; Shalon et al., *Genome Res.* (1996), 6:639-645; and Schena et al., *Proc. Natl. Acad. Sci. U.S.A.* (1995), 93:10539-11286); (iii) by masking (Maskos and Southern, *Nuc. Acids. Res.* (1992), 20:1679-1684) and (iv) by dot-blotting on a nylon or nitrocellulose hybridization membrane (see, e.g., Sambrook et al., Eds., 1989, Molecular Cloning: A Laboratory Manual, 2nd ed., Vol. 1-3, Cold Spring Harbor Laboratory (Cold Spring Harbor, N.Y.)). Probes may also be noncovalently immobilized on the substrate by hybridization to anchors, by means of magnetic beads, or in a fluid phase such as in microtiter wells or capillaries. The probe molecules are generally nucleic acids such as DNA, RNA, PNA, and cDNA but may also include proteins, polypeptides, oligosaccharides, cells, tissues and any permutations thereof which can specifically bind the target molecules.

The term "3'" generally refers to a region or position in a polynucleotide or oligonucleotide 3' (downstream) from another region or position in the same polynucleotide or oligonucleotide.

The term "5'" generally refers to a region or position in a polynucleotide or oligonucleotide 5' (upstream) from another region or position in the same polynucleotide or oligonucleotide.

The term "3'-DNA portion," "3'-DNA region," "3'-RNA portion," and "3'-RNA region," refer to the portion or region of a polynucleotide or oligonucleotide located towards the 3' end of the polynucleotide or oligonucleotide, and may or may not include the 3' most nucleotide(s) or moieties attached to the 3' most nucleotide of the same polynucleotide or oligonucleotide. The 3' most nucleotide(s) can be preferably from about 1 to about 20, more preferably from about 3 to about 18, even more preferably from about 5 to about 15 nucleotides.

The term "5'-DNA portion," "5'-DNA region," "5'-RNA portion," and "5'-RNA region," refer to the portion or region of a polynucleotide or oligonucleotide located towards the 5' end of the polynucleotide or

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oligonucleotide, and may or may not include the 5' most nucleotide(s) or moieties attached to the 5' most nucleotide of the same polynucleotide or oligonucleotide. The 5' most nucleotide(s) can be preferably from about 1 to about 20, more preferably from about 3 to about 18, even more preferably from about 5 to about 15 nucleotides.

As is well understood by those skilled in the art, a "tail" sequence of a primer is a sequence not hybridizable to the target sequence under conditions in which other region(s) or portion(s) of the primer hybridizes to the target.

Amplification methods of the invention

The following are examples of the amplification methods of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

Methods of generating multiple copies of (amplifying) an RNA sequence complementary to an RNA sequence of interest are provided. In some aspect, the amplification methods of the invention include a transcription step. In a first embodiment of these methods, linear nucleic acid amplification is achieved based on hybridizing a propromoter polynucleotide to a primer extension product to generate an intermediate product capable of driving transcription, whereby RNA transcripts comprising sequences complementary to an RNA sequence of interest are produced. In a second embodiment of these methods, exponential amplification is achieved by subjecting amplified RNA products generated in the process of the first embodiment of this method and in subsequent amplification to cyclical amplification.

In embodiments of the amplification methods of the invention which include a transcription step generally provide as follows: if a primer extension product that is to be transcribed comprises a propromoter sequence, a double stranded promoter region is generally generated by hybridizing a polynucleotide comprising a propromoter (also referred to herein as "propromoter polynucleotide") to the primer extension product. If a primer extension product does not comprise a desired propromoter sequence, the transcription step is

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generally dependent on the incorporation of an RNA polymerase propromoter sequence, by use of a propromoter polynucleotide such as a promoter sequence oligonucleotide (PTO). A propromoter polynucleotide such as the PTO can serve as a template for extension of a single stranded primer extension product and formation of a partial duplex comprising a double stranded promoter at one end. The ability to hybridize a single stranded primer extension product to the propromoter polynucleotide (such as a PTO) is generally achieved by creating a primer extension product with a defined 3' end sequence, which is complementary to the 3' end sequence of the propromoter polynucleotide.

In another aspect, the invention provides a method for generating multiple copies (amplifying) of a polynucleotide (DNA) sequence complementary to an RNA sequence of interest using a first primer and a composite primer.

One aspect of the methods of the invention includes the design of primers which are able to hybridize to RNA sequences, such as a plurality of RNA sequences, for initiation of primer extension and production of amplification substrates and products.

Methods of amplifying an RNA sequence of interest

Nucleic acid sequence amplification using a first primer and a propromoter polynucleotide, and a propromoter polynucleotide

The invention provides methods of generating multiple copies of the complementary sequence of an RNA sequence of interest by using a primer that comprises a sequence that when introduced into the amplification steps result in generation of an intermediate polynucleotide to which a propromoter polynucleotide can hybridize. The primer is designed to comprise a sequence the complement of which is hybridizable by a propromoter polynucleotide. Generally, this sequence is in the 5' portion of the primer. The sequence can be a sequence (the complement of which is hybridizable by the propromoter polynucleotide used) that is hybridizable to a target RNA, or a sequence (the complement of which is hybridizable by the propromoter polynucleotide used)

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that is not hybridizable to a target RNA. In some embodiments, linear amplification is achieved. In other embodiments wherein amplified RNA products are subjected to further rounds of amplification, cyclical, and thus exponential, amplification is achieved.

A schematic exemplary depiction of one embodiment of the linear methods of the invention is provided in Figure 1. A schematic depiction of one embodiment of the exponential amplification methods of the invention is provided in Figure 2. An embodiment of the linear amplification method of the invention illustrated in Figure 1 employs two oligonucleotides which are combined with the sample as shown in the figure: a) a first primer (labeled "4"), which can be composed of two portions, a 3' portion (labeled "A"), and a 5' portion (labeled "B"); and b) a second primer (labeled "5"). The exponential method of the invention as illustrated in Figures 2A-B employs three oligonucleotides which are combined with the sample as shown in the figure: a) a first primer (labeled "4") which can be composed of two portions, a 3' portion (labeled "A"); and a 5' portion (labeled "B"); b) a second primer (labeled "5"); and c) a propromoter polynucleotide, such as a promoter template oligonucleotide (PTO), (labeled "6").

The 3' portion of the first primer illustrated in Figures 1 and 2A can be designed in any of a number of ways (in terms of sequence), depending on which type, class, population, and/or species of RNA is desired to be amplified. In some embodiments, the 3' portion of the first primer, as illustrated in Figures 1 and 2A, comprises a sequence complementary to the poly-A tail of mRNA, and may further comprise additional random sequences (generally not complementary to a poly-A sequence) at the 3' end of the 3' portion. In other embodiments, the 3' portion of the first primer is a random primer comprising sequences which are hybridizable to a multiplicity of RNA species (which may range from 2 or more to many hundred or thousands or more). Random primers are known in the art, for example, they have been used extensively in the preparation of cDNA libraries using PCR-based procedures. As is well understood in the art, a "random primer" can refer to a primer that is a member of a population of primers (a plurality of random primers) which collectively are designed to hybridize to a desired and/or a

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significant number of target sequences. A random primer may hybridize at a plurality of sites on a nucleic acid sequence.

In other embodiments, the 3' portion of the first primer can comprise a sequence complementary or hybridizable to a specific RNA or family of RNAs (or portions thereof).

In some embodiments as illustrated in Figures 1 and 2A, the 5' portion of the first primer can be a sequence not hybridizable to the target sequence (under conditions in which the 3' portion hybridizes to RNA target), e.g., a sequence forming a "tail" when the primer is hybridized to a target. This "tail" sequence generally is incorporated into the first primer extension product (first strand cDNA), and the complement of this tail will be incorporated at the 3' end of the second primer extension product (second strand cDNA). Accordingly, in some embodiments, the first primer is a mixture of first primers which comprise the same 5' DNA portion and a multiplicity of 3' DNA portions selected to amplify a multiplicity (which can be small to very large) of RNA sequences of interest. In other embodiments, the 5' portion of the first primer can be hybridizable to the target RNA.

The second primer as illustrated in Figures 1 and 2A may comprise random sequences, which are known in the art, and that are complementary to sequences of a plurality of the first cDNA strands produced. The 3' end of the propromoter polynucleotide is preferably, but not necessarily, non-extendable. The 3' portion of the PTO generally comprises a sequence that is typically identical to the B sequence of the first primer 4.

Primer 2 as illustrated in Figures 1 and 2A can be, but is not necessarily, composed of DNA and can comprise two sections (interchangeably called "portions" or "regions"). The 3' portion of primer 2 can be selected for random priming of many, most and/or all possible mRNA sequences in a biological sample. Random primers are known in the art, for example, they have been used extensively in the preparation of cDNA libraries using PCR-based procedures. In some embodiments, the hybridizable sequence of the second primer is designed based on a known sequence of the desired binding site on a first strand cDNA. In other embodiments, the second primer comprises a strand switch oligonucleotide,

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described in U.S. Patent Nos. 5,962,271 and 5,962,272, which is hybridizable to the Cap sequences present on mRNA and causes the reverse transcriptase to switch from the mRNA template to the switch oligonucleotide, permitting generation of a second strand cDNA primed by the "switch oligonucleotide". Alternatively, a homopolymeric tail is added to the 3' terminus of the first primer extension product, and the second primer comprises the complement of the homopolymeric tail.

In some embodiments, the second primer comprises DNA. In other embodiments, the second primer consists of DNA. In other embodiments, as described herein, the second primer is a fragment of the target RNA, with the fragment being generated by cleavage of the RNA target.

The 5' portion of primer 2 can be a sequence which is not complementary and not substantially hybridizable to a specific target sequence, i.e., it would not hybridize (under conditions in which the 3' portion hybridizes to RNA target) and would constitute a tail. The "tail" sequence would generally be incorporated into the second primer extension product.

A promoter template oligonucleotide, 3 (PTO), can be designed as follows: the 3' end of the propromoter polynucleotide is preferably, but not necessarily, non-extendable. The 3' portion of the PTO generally comprises a sequence that is typically identical to the B sequence of the first primer 4. The 5'-most portion of the PTO is a promoter sequence for a DNA-dependent RNA polymerase, which, as described above, is used in certain embodiments of the amplification methods of the invention. Generally, the sequence between these two sections is designed for optimal transcription by the polymerase of choice. Criteria for selection and design of this sequence are known in the art.

As illustrated in Figure 1, one embodiment of the process of the linear amplification methods of the invention is as follows:

A) Formation of double stranded cDNA substrate from target RNA

1. Primer 4 binds to a target RNA by hybridization of its 3' end to the target to form complex I. The most commonly used initiation site for binding of the

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first primer to generate first strand cDNA is the 3' end poly-A sequence of mRNA and the immediate adjacent nucleotides. Binding of the primer to these immediate adjacent nucleotides can be achieved by including partially random sequences (other than sequence complementary to poly-A sequence of a target mRNA) at the 3' end of the primer. Criteria for primer designs for this purpose are known in the art, for example in the selection of primer sequences in the generation of libraries (including cDNA libraries). In cases where it is desired to produce libraries of sequences of a pre-defined family of mRNA, such as for example preparation of libraries from an immunoglobulin chain, the A sequence of the first primer could comprise a sequence which is well preserved in all members of the family of RNA from the immunoglobulin chain.

2. Primer extension along the RNA strand of complex I is carried out by an RNA-dependent DNA polymerase such as reverse transcriptase (labeled "RT"), to form an RNA/DNA hybrid duplex. RNase H degrades the RNA strand of the hybrid duplex to produce a first cDNA strand (labeled "I"). The RNase H activity may be supplied by the RNA-dependent DNA polymerase (such as reverse transcriptase) or may be provided in a separate enzyme. Reverse transcriptases useful for this method may or may not have RNase H activity.
3. A second primer (labeled "5") binds to II at a complementary site to form complex III. In the case where a plurality of RNA species are being amplified simultaneously, the second primers could be random primers that bind to II at random complementary sites of a plurality of cDNA species. When it is desired to generate a library of a known mRNA family, such as for example a library of a specific immunoglobulin chain, primer 5 may comprise a sequence which is complementary to a well conserved sequence in this family.
4. Primer extension along the first cDNA strand is carried out by a DNA-dependent DNA polymerase to generate a double stranded cDNA (labeled "IV") comprising at one end a duplex of sequence B (of the first primer) and its complement. The cDNAs are substrates for amplification according to the methods of the invention.

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5. The double stranded products IV are denatured (for example, by heat) to separate the two DNA strands. Various methods for strand separation could be employed for carrying out the methods of the invention. Strand V, which is of the same sense as the input (target) RNA, is a substrate for amplification, and comprises at its 5' end the sequence of the second primer (primer 5), and at its 3' end the sequence complementary to sequence B of the first primer (primer 4).

B) Linear isothermal amplification

1. A propromoter oligonucleotide (such as a PTO) binds to the 3' end of strand V by hybridization of its 3' end sequence B to its complementary sequence at the 3' end of V, to form complex VI.
2. A DNA-dependent DNA polymerase extends the 3' end of the sense cDNA strand (strand V) in complex VI to form the partial duplex VII, comprising a double stranded RNA polymerase promoter at one end.
3. An RNA polymerase binds to the double stranded promoter and transcribes the cDNA strand of complex VII to produce multiple copies of single stranded anti-sense RNA products, VIII. These antisense RNA products can also serve as substrates for exponential amplification.

One embodiment of the exponential amplification methods of the invention is illustrated in Figures 2A-B. In this embodiment, subsequent to the generation of antisense RNA products in the linear amplification steps, the following steps are performed.

C) Exponential isothermal amplification

1. Primer 5 binds to the 3' end of the RNA products VIII to form complex IX.
2. Primer extension along the RNA strand is carried out by an RNA-dependent DNA polymerase to form an RNA/DNA hybrid duplex, X.

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3. RNase H degrades the RNA strand of complex X to produce single stranded DNA copies of the RNA products. A propromoter polynucleotide such as a PTO hybridizes to the 3' end of the single stranded DNA to form complex XI.
4. The 3' end of the DNA strand in complex XI is extended along the propromoter polynucleotide (PTO) to form a partial duplex XII, comprising a double stranded promoter sequence at one end. This product is the same as VII, and is a substrate for RNA polymerase for the generation of multiple copies of the RNA products, as in step b(3) above, thus producing a cyclical process for the exponential amplification of a target RNA.

Nucleic acid sequence amplification using a first primer, a composite primer, denaturation and strand displacement

The invention provides methods of amplifying an RNA sequence of interest by using a first primer and a composite primer, denaturation, and strand displacement. Amplification can be achieved isothermally, though every step is not necessarily conducted at the same temperature. Amplified products are single stranded DNA comprising sequences complementary to the RNA sequence of interest in the target RNA.

A schematic description of one embodiment of the composite primer, second primer and strand displacement-based methods of the invention is given in Figures 3A and B. The methods involve the following steps: (a) formation of a second strand cDNA which is the same sense as the input RNA (as described herein, and one embodiment of which is illustrated in Figure 1); and (b) linear amplification of the complement of a second strand cDNA strand by primer extension from a composite primer along the second strand cDNA and strand displacement. See Kurn, U.S. Patent No. 6,251,639 B1.

The embodiment illustrated in Figures 3A and 3B employs three oligonucleotides: a first primer which can be composed of two portions, a 3' portion (labeled "A"); and a 5' portion (labeled "B"), (labeled 1), used for formation of first strand cDNA; a second primer , used for the formation of the second strand cDNA (which may be an exogenously added second primer or one or more fragments of target RNA that remains hybridized to the first strand cDNA

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following RNase H treatment); and a composite primer used for linear isothermal amplification. The composite primer comprises a5' RNA portion and a DNA portion. The first and second primers used in this aspect of the invention may comprise any first and second primer described herein (including a second primer comprising one or more fragments .

The composite primer illustrated in Figure 3A and B comprises a sequence capable of hybridizing to the second strand cDNA, and most often comprises a sequence hybridizable to the defined 3'-portion of the second strand cDNA (that is the complement of the first primer sequence). The composite primer may additionally comprise sequences not hybridizable to the second strand cDNA under conditions which the composite primer hybridizes, such that a tail is formed.

As illustrated in Figures 3A and B, the composite primer comprises a DNA portion at its 3' end, and an RNA portion at its 5' end. As discussed herein, it is also possible to employ a composite primer in which the 3' DNA portion is followed, in the direction of its 5', by an RNA portion, which is followed by a portion which is DNA. The length of each of these sections is generally determined for maximum efficiency of the amplification. Only the two-portion (i.e., 3'-DNA-RNA-5') composite primer is shown in Figures 3A and B.

As illustrated in Figures 3A and B, in one embodiment, the process of the amplification methods of the invention resulting in generation of DNA products comprising sequences complementary to an RNA sequence(s) of interest based on RNA is as follows:

A) Formation of a single stranded cDNA substrate for amplification using a composite primer

1. A primer binds to a target RNA by hybridization of its 3' end to the target.
2. Primer extension along the RNA strand is carried out by an RNA-dependent DNA polymerase such as reverse transcriptase, to form an RNA/DNA hybrid duplex. RNase H degrades the RNA strand of the hybrid duplex to produce a first cDNA strand (labeled "I"). The RNase H activity may be supplied by the RNA-dependent DNA polymerase (such as reverse transcriptase) or may be

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provided in a separate enzyme. Reverse transcriptases useful for this method may or may not have RNase H activity.

3. Primer extension along the first cDNA strand is carried out by a DNA-dependent DNA polymerase (not illustrated in Figure 3A) to generate a double stranded cDNA , forming a complex of first and second strand cDNAs (wherein the second strand cDNA comprises a 3' end portion that is the complement of the first primer sequence), as shown in Figure 3A.
4. The complex of first and second strand cDNAs is then denatured to form single stranded first strand cDNA and second strand cDNA, as shown in Figure 3B. The single stranded second strand cDNA is the substrate for isothermal amplification using a composite primer and strand displacement as follows.

B) Generation of double stranded cDNA comprising an RNA/DNA heteroduplex

1. A composite primer comprising a 5'-RNA portion and a DNA portion hybridizes to the 3'-portion of the second strand cDNA, generally to the 3'-portion of the second strand cDNA, and is extended along the second strand cDNA by a DNA polymerase to form a double stranded complex comprising an RNA/DNA hybrid portion at one end of the complex.

C) Isothermal linear amplification

1. An agent, such as an enzyme, which cleaves RNA from an RNA/DNA hybrid (such as RNase H) cleaves RNA sequence from the hybrid, leaving a sequence on the second strand cDNA available for binding by another composite primer.
2. Composite primer binds by hybridization of the RNA portion to the single stranded DNA end on the second strand cDNA, which is complementary to it. The 3' DNA sequence of primer is not hybridized.
3. The 3' end of bound composite primer, and the 5' end of the DNA strand immediately upstream are the same, and would compete for hybridization to the opposite strand. Without wishing to be bound by theory, the high affinity

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of the DNA polymerase to hybridized 3' end of a primer would be expected to push the equilibrium of the two competing structures towards hybridization of the 3' end of the new primer and displacement of the 5' end of the previous primer extension product. Primer extension along the second strand (sense) cDNA strand results in displacement of the previous second strand cDNA, and formation of a double stranded cDNA having, at one end, an RNA/DNA hybrid composed of sequence B and its complement

4. The RNA segment of the hybrid is degraded by agent, such as RNase H, which results in the formation of a single stranded 3' end to which a new composite primer can be bound by its 5' portion.
5. The process of hybridization of the 3' end sequence of the bound composite primer, by displacement of the 5'-most end of the previous primer extension product in the duplex, primer extension and displacement of the previous product continues , and results in the accumulation of multiple copies of anti-sense single stranded DNA products.

In some embodiments, subsequent to the generation of antisense RNA products in the linear amplification steps, the following steps are performed:

D) Exponential isothermal amplification

1. A propromoter oligonucleotide (such as a PTO) binds to the 3' end of anti-sense single stranded DNA products.
2. Primer extension along the RNA strand is carried out by an RNA-dependent DNA polymerase to form an RNA/DNA hybrid duplex. This product is the same is a substrate for RNA polymerase for the generation of multiple copies of sense RNA products. As described herein, extension of the propromoter polynucleotide may or may not be required to effect creation of a propromoter for transcription.

Components and reaction conditions used in the methods of the invention

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Template nucleic acid

The RNA target to be amplified includes RNAs from any source in purified or unpurified form, which can be RNA such as total RNA, tRNA, mRNA, rRNA, mitochondrial RNA, chloroplast RNA, DNA-RNA hybrids, or mixtures thereof, from any source and/or species, including human, animals, plants, and microorganisms such as bacteria, yeasts, viruses, viroids, molds, fungi, plants, and fragments thereof. RNAs can be obtained and purified using standard techniques in the art. Amplification of a DNA target would require initial transcription of the DNA target into RNA form, which can be achieved by techniques (such as expression systems) known in the art. Amplification of a DNA-RNA hybrid would require denaturation of the hybrid to obtain a ssRNA, or denaturation followed by transcription of the DNA strand to obtain an RNA. The target RNA can be only a minor fraction of a complex mixture such as a biological sample and can be obtained from various biological material by procedures well known in the art.

The target RNA can be known or unknown and may contain more than one desired specific nucleic acid sequence of interest, each of which may be the same or different from each other. Therefore, the amplification process is useful not only for producing large amounts of one specific nucleic acid sequence, but also for amplifying simultaneously more than one different specific nucleic acid sequence located on the same or different nucleic acid molecules.

The initial step of the amplification of a target RNA sequence is rendering the target single stranded. Denaturation may be carried out to remove secondary structure present in a RNA target molecule. The denaturation step may be thermal denaturation or any other method known in the art.

First primer

The first primer is a primer that comprises a sequence (which may or may not be the whole of the primer) that is hybridizable (under a given set of conditions) to the target RNA. This sequence can be based on a specific sequence

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of the target, or a random sequence (in some embodiments, the first primer is a random primer). It can also be based on a general, more universal sequence known to be present in an RNA species of interest, such as the poly-A sequence found in mRNA. In some embodiments, the primer may comprise a sequence complementary to a poly-A sequence, and may further comprise a random sequence 3' to said sequence complementary to a poly-A sequence. In some embodiments, the primer comprises a sequence, preferably at the 5' end, that is not hybridizable (under a given set of conditions) to a target RNA. In addition, the sequence that is capable of hybridizing to the target RNA may comprise a sequence complementary to the poly-A tail of mRNA, and may further comprise additional random sequences (generally not complementary to a poly-A sequence) at the 3' end of the 3' portion.

Random primers are well known in the art, and include at least the following: primers hybridizable to two or more sequences in a sample; and primers comprising poly-T sequences that are hybridizable to a multiplicity of RNAs in a sample (such as all mRNA). For convenience, a single random composite primer is discussed above. However, it is understood that the term "random primer" can refer to a primer that is a member of a population of primers which are collectively designed to a desired and/or significant population of target sequences.

It is also understood that the amplification of a plurality of mRNA species in a single reaction mixture may, but not necessarily, employ a multiplicity, or a large multiplicity of primers. Thus, the invention contemplates the use of a multiplicity of different composite primers (random or non-random) when amplifying a plurality of mRNA species in a single reaction mixture.

To achieve hybridization to a target nucleic acid (which, as is well known and understood in the art, depends on other factors such as, for example, ionic strength and temperature), the sequence of the primer that is hybridizable to the target RNA is preferably of at least about 60%, more preferably at least about 75%, even more preferably at least about 90%, and most preferably at least about 95% complementarily to the target nucleic acid.

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In some embodiments, the first primer comprises a 5' sequence (which generally includes the 5' most nucleotide) the complement of which is hybridizable by a propromoter polynucleotide. This sequence enables the creation of a defined end sequence for the 5' end of the first primer extension product (and thus, subsequently the 3' end of the second/third primer extension product). Having a defined end sequence is particularly advantageous with respect to hybridization of a propromoter polynucleotide to the 3' end of the second and third primer extension products in subsequent steps. Thus, in these embodiments, the first primer comprises a sequence that when introduced into the amplification steps of the methods of the invention results in generation of an intermediate polynucleotide to which a propromoter polynucleotide can hybridize. In some of these embodiments, the 5' sequence the complement of which is hybridizable by a propromoter polynucleotide is hybridizable (under a given set of conditions) to a target RNA when the primer is hybridized to the target RNA. In other embodiments, the 5' sequence the complement of which is hybridizable by a propomoter polynucleotide is not hybridizable (under a given set of conditions) to a target RNA when the primer is hybridized to the target RNA (thus constituting a tail when the 3' sequence of the primer is hybridized to the target).

In one embodiment, the first primer comprises DNA. In another embodiment, the first primer comprises RNA. In yet another embodiment, the first primer comprises DNA and RNA.

Second primer

The second primer in the methods of the invention comprises a sequence (which may or may not be the whole of the primer) that is hybridizable (under a given set of conditions) to a first primer extension product at a site on the first primer extension product such that the second primer extension product would include the RNA sequence of interest. In some embodiments, the hybridizable sequence of the second primer is designed based on a known sequence of the desired binding site on a first primer extension product. In other embodiments, the hybridizable sequence is based on random sequences known in the art to be

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suitable for random priming of a plurality of RNA species. In some embodiments, a second primer comprises a sequence, preferably at the 5' end, that is not hybridizable (under a given set of conditions) to a first primer extension product. In other embodiments, the second primer comprises a strand switch oligonucleotide, described in U.S. Patent Nos. 5,962,271 and 5,962,272, which is hybridizable to the Cap sequences present on mRNA and causes the reverse transcriptase to switch from the mRNA template to the switch oligonucleotide, permitting generation of a second strand cDNA primed by the "switch oligonucleotide". Alternatively, a homopolymeric tail is added to the 3' terminus of the first primer extension product, and the second primer comprises the complement of the homopolymeric tail.

In one embodiment, the second primer comprises DNA. In another embodiment, the second primer consists of DNA. In another embodiment, the second primer comprises RNA. In yet another embodiment, the second primer comprises DNA and RNA.

In some embodiments, the second primer is provided by self priming (for example, by a hairpin loop) at the 3' end of the first primer extension product. In these embodiments, a sequence at the 3' end of the first primer extension product hybridizes to another sequence in the first primer extension product, for example as described in U.S. Pat. No. 6,132,997. In these embodiments, said sequence at the 3' of the first primer extension product is generally cleaved (for example, with an S1 nuclease) following its hybridization to the first primer extension product and/or its extension along the first primer extension product. See U.S. Pat. No. 6,132,997.

In some embodiments, the second primer is provided by a target RNA fragment. Such a target RNA fragment can be generated as a result of incomplete degradation of a target RNA in a complex of target RNA and first primer extension product by an enzyme that cleaves RNA in an RNA/DNA hybrid, such that one or more RNA fragments remain bound to the first primer extension product.

To achieve hybridization to a first primer extension product (which, as is well known and understood in the art, depends on other factors such as, for

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example, ionic strength and temperature), the sequence of the second primer that is hybridizable to the first primer extension product is preferably of at least about 60%, more preferably at least about 75%, even more preferably at least about 90%, and most preferably at least about 95% complementarity to the first primer extension product.

Third primer

The third primer in the methods of the invention comprises a sequence (which may or may not be the whole of the primer) that is hybridizable (under a given set of conditions) to the RNA transcript generated from the second primer extension product (as the template) at a site on the RNA transcript such that the third primer extension product would include the RNA sequence of interest, if present. In some embodiments, the hybridizable sequence of the third primer is designed based on a known sequence of the desired binding site on an RNA transcript. In other embodiments, the hybridizable sequence is based on random sequences known in the art to be suitable for random priming of a plurality of RNA species. In some embodiments, the third primer comprises a sequence, preferably at the 5' end, that is not hybridizable (under a given set of conditions) to an RNA transcript.

To achieve hybridization to an RNA transcript (which, as is well known and understood in the art, depends on other factors such as, for example, ionic strength and temperature), the sequence of the third primer that is hybridizable to the RNA transcript is preferably of at least about 60%, more preferably at least about 75%, even more preferably at least about 90%, and most preferably at least about 95% complementarity to the RNA transcript.

In one embodiment, the third primer comprises DNA. In another embodiment, the third primer comprises RNA. In yet another embodiment, the third primer comprises DNA and RNA.

In some embodiments, the third primer is provided by self priming (for example, by a hairpin loop) at the 3' end of an RNA transcript. In these embodiments, a sequence at the 3' end of the RNA transcript hybridizes to

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another sequence in the RNA transcript itself. In these embodiments, said sequence at the 3' of the RNA transcript is generally cleaved following its hybridization to the RNA transcript and/or its extension along the RNA transcript.

Composite primer

In some embodiments, the methods of the invention employ a composite primer that is composed of RNA and DNA portions. The composite primer is designed such that subsequent displacement of the primer extension product by binding of a new (additional) composite primer and the extension of the new primer by the polymerase can be achieved. In addition, cleavage of the RNA portion of the primer extension product leads to generation of amplification product which is not a substrate for amplification by the composite primer.

The composite primer illustrated in Figure 3 comprises sequences capable of hybridizing to the second strand cDNA, and most often comprises sequences hybridizable to the defined 3'-portion of the second strand cDNA (that is the complement of the first primer sequence). The composite primer may comprise all or a portion of the sequence of the first primer. The composite primer may additionally comprise sequences not hybridizable to the second strand cDNA such that a tail is formed.

It is also understood that the amplification of a plurality of mRNA species in a single reaction mixture may, but not necessarily, employ a multiplicity, or a large multiplicity of primers. Thus, the invention contemplates the use of a multiplicity of different composite primers (random or non-random) when amplifying a plurality of mRNA species in a single reaction mixture.

A composite primer comprises at least one RNA portion that is capable of (a) binding (hybridizing) to a sequence on the second strand cDNA product independent of hybridization of the DNA portion(s) to a sequence on the same extension product; and (b) being cleaved with a ribonuclease when hybridized to the second strand cDNA. The composite primers bind to the second strand cDNA to form a partial heteroduplex in which only the RNA portion of the primer is cleaved upon contact with a ribonuclease such as RNase H, while the second strand cDNA remains intact, thus enabling annealing of another composite primer.

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The composite primers also comprise a 3' DNA portion that is capable of hybridization to a sequence on the second strand cDNA such that its hybridization to the cDNA is favored over that of the nucleic acid strand that is displaced from the second strand cDNA by the DNA polymerase. Such primers can be rationally designed based on well known factors that influence nucleic acid binding affinity, such as sequence length and/or identity, as well as hybridization conditions. In a preferred embodiment, hybridization of the 3' DNA portion of the composite primer to its complementary sequence in the second strand cDNA is favored over the hybridization of the homologous sequence in the 5' end of the displaced strand to the second strand cDNA.

Generation of primers suitable for extension by polymerization is well known in the art, such as described in PCT Pub. No. WO99/42618 (and references cited therein). The composite primer comprises a combination of RNA and DNA (see definition above), with the 3'-end nucleotide being a nucleotide suitable for nucleic acid extension. The 3'-end nucleotide can be any nucleotide or analog that when present in a primer, is extendable by a DNA polymerase. Generally, the 3'-end nucleotide has a 3'-OH. Suitable primers include those that comprise at least one portion of RNA and at least one portion of DNA. For example, composite primers can comprise a 5'-RNA portion and a 3'-DNA portion (in which the RNA portion is adjacent to the 3'-DNA portion); or 5'- and 3'-DNA portions with an intervening RNA portion. Accordingly, in one embodiment, the composite primer comprises a 5' RNA portion and a 3'-DNA portion, preferably wherein the RNA portion is adjacent to the 3'-DNA portion. In another embodiment, the composite primer comprises 5'- and 3'-DNA portions with at least one intervening RNA portion (i.e., an RNA portion between the two DNA portions). In yet another embodiment, the composite primer of the invention comprises a 3'-DNA portion and at least one intervening RNA portion (i.e., an RNA portion between DNA portions).

The length of an RNA portion in a composite primer comprising a 3'-DNA portion and an RNA portion can be preferably from about 1 to about 50, more preferably from about 3 to about 20, even more preferably from about 4 to about 15, and most preferably from about 5 to about 10 nucleotides. In some

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embodiments of a composite primer comprising a 3'-DNA portion and an RNA portion, an RNA portion can be at least about any of 1, 3, 4, 5 nucleotides, with an upper limit of about any of 10, 15, 20, 25, 3, 50 nucleotides.

The length of the 5'-RNA portion in a composite primer comprising a 5'-RNA portion and a 3'-DNA portion can be preferably from about 3 to about 50 nucleotides, more preferably from about 5 to about 20 nucleotides, even more preferably from about 7 to about 18 nucleotides, preferably from about 8 to about 17 nucleotides, and most preferably from about 10 to about 15 nucleotides. In other embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, the 5'-RNA portion can be at least about any of 3, 5, 7, 8, 10 nucleotides, with an upper limit of about any of 15, 17, 18, 20, 50 nucleotides.

In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion further comprising non-5'-RNA portion(s), a non-5'-RNA portion can be preferably from about 1 to about 7 nucleotides, more preferably from about 2 to about 6 nucleotides, and most preferably from about 3 to about 5 nucleotides. In certain embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion further comprising non-5'-RNA portion(s), a non-5'-RNA portion can be at least about any of 1, 2, 3, 5, with an upper limit of about any of 5, 6, 7, 10 nucleotides.

In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the length of the 5'-RNA portion can be preferably from about 3 to about 50 nucleotides, more preferably from about 5 to about 20 nucleotides, even more preferably from about 7 to about 18 nucleotides, preferably from about 8 to about 17 nucleotides, and most preferably from about 10 to about 15 nucleotides. In certain embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the 5'-RNA portion can be at least about any of 3, 5, 7, 8, 10 nucleotides, with an upper limit of about any of 15, 17, 18, 20, 50 nucleotides.

The length of an intervening RNA portion in a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion can be preferably from about 1 to about 7 nucleotides, more preferably from about

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2 to about 6 nucleotides, and most preferably from about 3 to about 5 nucleotides. In some embodiments of a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion, an intervening RNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 5, 6, 7, 10 nucleotides. The length of an intervening RNA portion in a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion can be preferably from about 1 to about 7 nucleotides, more preferably from about 2 to about 6 nucleotides, and most preferably from about 3 to about 5 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, an intervening RNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 5, 6, 7, 10 nucleotides. In a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, further comprising a 5'-RNA portion, the 5'-RNA portion can be preferably from about 3 to about 25 nucleotides, more preferably from about 5 to about 20 nucleotides, even more preferably from about 7 to about 18 nucleotides, preferably from about 8 to about 17 nucleotides, and most preferably from about 10 to about 15 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, further comprising a 5'-RNA portion, the 5'-RNA portion can be at least about any of 3, 5, 7, 8, 10 nucleotides, with an upper limit of about any of 15, 17, 18, 20 nucleotides.

The length of the 3'-DNA portion in a composite primer comprising a 3'-DNA portion and an RNA portion can be preferably from about 1 to about 20, more preferably from about 3 to about 18, even more preferably from about 5 to about 15, and most preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and an RNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides.

The length of the 3'-DNA portion in a composite primer comprising a 5'-RNA portion and a 3'-DNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7

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to about 12 nucleotides. In some embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, the 3' DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides.

In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, further comprising non-3'-DNA portion(s), a non-3'-DNA portion can be preferably from about 1 to about 10 nucleotides, more preferably from about 2 to about 8 nucleotides, and most preferably from about 3 to about 6 nucleotides. In some embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, further comprising non-3'-DNA portion(s), a non-3'-DNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 6, 8, 10, 12 nucleotides.

In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the length of the 3'-DNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In certain embodiments of the primer comprising a 5'-RNA portion and a 3'-DNA portion in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides.

The length of a non-3'-DNA portion in a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion can be preferably from about 1 to about 10 nucleotides, more preferably from about 2 to about 8 nucleotides, and most preferably from about 3 to about 6 nucleotides. In some embodiments of a primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion, a non-3'-DNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 6, 8, 10, 12 nucleotides.

The length of the 3'-DNA portion in a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most

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preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides.

The length of a non-3'-DNA portion (i.e., any DNA portion other than the 3'-DNA portion) in a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion can be preferably from about 1 to about 10 nucleotides, more preferably from about 2 to about 8 nucleotides, and most preferably from about 3 to about 6 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, a non-3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 6, 8, 10, 12 nucleotides. The length of the 3'-DNA portion in a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides. It is understood that the lengths for the various portions can be greater or less, as appropriate under the reaction conditions of the methods of this invention.

In some embodiments, the 5'-DNA portion of a composite primer includes the 5'-most nucleotide of the primer. In some embodiments, the 5'-RNA portion of a composite primer includes the 5' most nucleotide of the primer. In other embodiments, the 3'-DNA portion of a composite primer includes the 3' most nucleotide of the primer. In other embodiments, the 3'-DNA portion is adjacent to the 5'-RNA portion and includes the 3' most nucleotide of the primer (and the 5'-RNA portion includes the 5' most nucleotide of the primer).

The total length of the composite primer can be preferably from about 10 to about 50 nucleotides, more preferably from about 15 to about 30 nucleotides,

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and most preferably from about 20 to about 25 nucleotides. In some embodiments, the length can be at least about any of 10, 15, 20, 25 nucleotides, with an upper limit of about any of 25, 30, 50, 60 nucleotides. It is understood that the length can be greater or less, as appropriate under the reaction conditions of the methods of this invention.

To achieve hybridization to a target nucleic acid (which, as is well known and understood in the art, depends on other factors such as, for example, ionic strength and temperature), the portion of the primer that is hybridizable to the target RNA is preferably of at least about 60%, more preferably at least about 75%, even more preferably at least about 90%, and most preferably at least about 95% complementarity to the target nucleic acid.

Polynucleotide comprising a propromoter and a region which hybridizes to a primer extension product

The methods of the invention employ a propromoter polynucleotide comprising a propromoter and a region which hybridizes to a primer extension product. In some embodiments, the propromoter polynucleotide is provided as a PTO, as described in greater detail below.

Propromoter template oligonucleotide

In some embodiments, the methods employ a promoter sequence for transcription which is provided by a propromoter template oligonucleotide (PTO).

A PTO for use in the methods and compositions of the invention is a single-stranded polynucleotide, generally DNA, comprising a propromoter sequence that is designed for formation of a double stranded promoter of an RNA polymerase, and a portion capable of hybridizing to the 3' end of a primer extension product. In a preferred embodiment, the propromoter sequence is located in the 5' portion of the oligonucleotide and the hybridizing sequence is located in the 3' portion of the oligonucleotide. In one embodiment, and most typically, the promoter and hybridizing sequences are different sequences. In another embodiment, the promoter and hybridizing sequences overlap in sequence identity. In yet another embodiment, the promoter and hybridizing sequences are

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the same sequence, and thus are in the same location on the PTO. In the embodiments wherein hybridization of the PTO to the primer extension product results in a duplex comprising an overhang (the 5' end of the PTO that does not hybridize to the primer extension product, typically comprising all or part of the propromoter sequence), DNA polymerase fills in the overhang to create a double stranded promoter capable of effecting transcription by a suitable RNA polymerase.

Promoter sequences that allow transcription of a template DNA are known in the art and have been discussed above. Preferably, the promoter sequence is selected to provide optimal transcriptional activity of the particular RNA polymerase used. Criteria for such selection, i.e., a particular promoter sequence particularly favored by a particular RNA polymerase, is also known in the art. For example, the sequences of the promoters for transcription by T7 DNA dependent RNA polymerase and SP6 are known in the art. The promoter sequence can be from a prokaryotic or eukaryotic source.

In some embodiments, the PTO comprises an intervening sequence between a propromoter sequence and a portion capable of hybridizing to the 3' end of the primer extension product. Suitable length of the intervening sequence can be empirically determined, and can be at least about 1, 2, 4, 6, 8, 10, 12, 15 nucleotides. Suitable sequence identity of the intervening sequence can also be empirically determined, and the sequence is designed to preferably, but not necessarily, enhance degree of amplification as compared to omission of the sequence. In one embodiment, the intervening sequence is a sequence that is designed to provide for enhanced, or more optimal, transcription by the RNA polymerase used. Generally, the sequence is not related (i.e., it does not substantially hybridize) to a primer extension product. More optimal transcription occurs when transcriptional activity of the polymerase from a promoter that is operatively linked to said sequence is greater than from a promoter that is not so linked. The sequence requirements for optimal transcription are generally known in the art as previously described for various DNA dependent RNA polymerases, such as in U.S. Pat. Nos. 5766849 and 5654142, and can also be empirically determined.

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In another embodiment, the PTO comprises a sequence that is 5' to the propromoter sequence, i.e., the PTO comprises additional nucleotides (which may or may not be transcriptional regulatory sequences) located 5' to the propromoter sequence. Generally, but not necessarily, the sequence is not hybridizable (under a given set of conditions) to the primer extension product.

In one embodiment, the PTO cannot function efficiently as a primer for nucleic acid extension. Techniques for blocking the primer function of the PTO include any that prevent addition of nucleotides to the 3' end of the PTO by a DNA polymerase. Such techniques are known in the art, including, for example, substitution or modification of the 3' hydroxyl group, or incorporation of a modified nucleotide, such as a dideoxynucleotide, in the 3'-most position of the PTO that is not capable of anchoring addition of nucleotides by a DNA polymerase. It is possible to block the 3' end using a label, or a small molecule which is a member of a specific binding pair, such as biotin. It is also possible to render the 3' end non-extendable by addition of nucleotides which cannot hybridize to a primer extension product, either due to non-complementarity or due to structural modifications which do not support hydrogen bonding. In other embodiments, the PTO is not blocked.

The length of the portion of the PTO that hybridizes to a primer extension product of interest is preferably from about 5 to about 50 nucleotides, more preferably from about 10 to about 40 nucleotides, even more preferably from about 15 to about 35 nucleotides, and most preferably from about 20 to 30 nucleotides. In some embodiments, the hybridizing portion is at least about any of the following: 3, 5, 10, 15, 20; and less than about any of the following: 30, 40, 50, 60. The complementarity of the hybridizing portion is preferably at least about 25%, more preferably at least about 50%, even more preferably at least about 75%, and most preferably at least about 90%, to its intended binding sequence on the primer extension product of interest.

DNA polymerase, ribonuclease and RNA polymerase

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The amplification methods of the invention employ the following enzymes: an RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase, a ribonuclease such as RNase H, and a DNA-dependent RNA polymerase. One or more of these activities may be found and used in a single enzyme. For example, RNase H activity may be supplied by an RNA-dependent DNA polymerase (such as reverse transcriptase) or may be provided in a separate enzyme. Reverse transcriptases useful for this method may or may not have RNase H activity.

One aspect of the invention is the formation of double stranded cDNA from a primer-RNA complex. This process generally utilizes the enzymatic activities of an RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase and a ribonuclease activity.

RNA-dependent DNA polymerases for use in the methods and compositions of the invention are capable of effecting extension of a primer according to the methods of the invention. Accordingly, a preferred RNA-dependent RNA polymerase is one that is capable of extending a nucleic acid primer along a nucleic acid template that is comprised at least predominantly of ribonucleotides. Suitable RNA-dependent DNA polymerases for use in the methods and compositions of the invention include reverse transcriptase. Many reverse transcriptases, such as those from avian myeoloblastosis virus (AMV-RT), and Moloney murine leukemia virus (MMLV-RT) comprise more than one activity (for example, polymerase activity and ribonuclease activity) and can function in the formation of the double stranded cDNA molecules. However, in some instances, it is preferable to employ a reverse transcriptase which lacks the RNase H activity. Reverse transcriptase devoid of RNase H activity are known in the art, including those comprising a mutation of the wild type reverse transcriptase where the mutation eliminates the RNase H activity. In these cases, the addition of an RNase H from other sources, such as that isolated from *E. coli*, can be employed for the formation of the double stranded cDNA.

DNA-dependent DNA polymerases for use in the methods and compositions of the invention are capable of effecting extension of a primer according to the methods of the invention. Accordingly, a preferred polymerase is

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one that is capable of extending a nucleic acid primer along a nucleic acid template that is comprised at least predominantly of deoxynucleotides. The formation of the double stranded cDNA can be carried out by reverse transcriptase which comprises both RNA-dependent DNA polymerase and DNA-dependent DNA polymerase activities. Preferably, the DNA polymerase has high affinity for binding at the 3'-end of an oligonucleotide hybridized to a nucleic acid strand. Preferably, the DNA polymerase does not possess substantial nicking activity. Preferably, the polymerase has little or no 5'->3' exonuclease activity so as to minimize degradation of primer, or primer extension polynucleotides. Generally, this exonuclease activity is dependent on factors such as pH, salt concentration, whether the template is double stranded or single stranded, and so forth, all of which are familiar to one skilled in the art. Mutant DNA polymerases in which the 5'->3' exonuclease activity has been deleted, are known in the art and are suitable for the amplification methods described herein. Preferably, the DNA polymerase has little to no proofreading activity.

Suitable DNA polymerases for use in the methods and compositions of the invention include those disclosed in U.S. Pat. Nos. 5648211 and 5744312, which include exo$^-$ Vent (New England Biolabs), exo$^-$ Deep Vent (New England Biolabs), Bst (BioRad), exo$^-$ Pfu (Stratagene), Bca (Panvera), sequencing grade Taq (Promega), and thermostable DNA polymerases from thermoanaerobacter thermohydrosulfuricus.

An agent that cleaves RNA in an RNA/DNA hybrid (e.g. ribonuclease) is used in the methods and compositions of the invention. Preferably, the agent, which can be ribonuclease, cleaves ribonucleotides regardless of the identity and type of nucleotides adjacent to the ribonucleotide to be cleaved. It is preferred that the agent (e.g. ribonuclease) cleaves independent of sequence identity. Examples of suitable ribonucleases for the methods and compositions of the invention are well known in the art, including ribonuclease H (RNase H), including Hybridase.

The DNA-dependent RNA polymerase for use in the methods and compositions of the invention are known in the art. Either eukaryotic or prokaryotic polymerases may be used. Examples include T7, T3 and SP6 RNA

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polymerases. Generally, the RNA polymerase selected is capable of transcribing from the promoter sequence provided by the propromoter polynucleotides as described herein. Generally, the RNA polymerase is a DNA-dependent polymerase, which is preferably capable of transcribing from a single stranded DNA template so long as the promoter region is double stranded.

In general, the enzymes used included in the methods and compositions of the invention should not produce substantial degradation of the nucleic acid components of said methods and compositions.

Reaction conditions and detection

Appropriate reaction media and conditions for carrying out the methods of the invention are those that permit nucleic acid amplification according to the methods of the invention. Such media and conditions are known to persons of skill in the art, and are described in various publications, such as U.S. Pat. Nos. 5,554,516; 5,716,785; 5,130,238; 5,194,370; 6,090,591; 5,409,818; 5,554,517; 5,169,766; 5,480,784; 5,399,491; 5,679,512; and PCT Pub. No. WO99/42618. For example, a buffer may be Tris buffer, although other buffers can also be used as long as the buffer components are non-inhibitory to enzyme components of the methods of the invention. The pH is preferably from about 5 to about 11, more preferably from about 6 to about 10, even more preferably from about 7 to about 9, and most preferably from about 7.5 to about 8.5. The reaction medium can also include bivalent metal ions such as Mg^{2+} or Mn^{2+}, at a final concentration of free ions that is within the range of from about 0.01 to about 15 mM, and most preferably from about 1 to 10 mM. The reaction medium can also include other salts, such as KCl or NaCl, that contribute to the total ionic strength of the medium. For example, the range of a salt such as KCl is preferably from about 0 to about 125 mM, more preferably from about 0 to about 100 mM, and most preferably from about 0 to about 75 mM. The reaction medium can further include additives that could affect performance of the amplification reactions, but that are not integral to the activity of the enzyme components of the methods.

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Such additives include proteins such as BSA, single stranded binding protein (for example, T4 gene 32 protein), and non-ionic detergents such as NP40 or Triton. Reagents, such as DTT, that are capable of maintaining enzyme activities can also be included. Such reagents are known in the art. Where appropriate, an RNase inhibitor (such as Rnasin) that does not inhibit the activity of the RNase employed in the method can also be included. Any aspect of the methods of the invention can occur at the same or varying temperatures. Preferably, the amplification reactions (particularly, primer extension and transcription; and generally not the step of denaturing the complex of first and second primer extension products) are performed isothermally, which substantially avoids the cumbersome thermocycling process. The amplification reaction is carried out at a temperature that permits hybridization of the oligonucleotides (primer, and/or PTO) of the invention to the template polynucleotide and that does not substantially inhibit the activity of the enzymes employed. The temperature can be in the range of preferably about 25°C to about 85°C, more preferably about 30°C to about 80°C, and most preferably about 37°C to about 75°C. The temperature for the transcription steps can be lower than the temperature(s) for the preceding steps. The temperature of the transcription steps can be in the range of preferably about 25°C to about 85°C, more preferably about 30°C to about 75°C, and most preferably about 37°C to about 70°C.

Nucleotide and/or nucleotide analogs, such as deoxyribonucleoside triphosphates, that can be employed for synthesis of the primer extension products in the methods of the invention are provided in the amount of from preferably about 50 to about 2500 μM, more preferably about 100 to about 2000 μM, even more preferably about 200 to about 1700 μM, and most preferably about 250 to about 1500 μM. Nucleotides and/or analogs, such as ribonucleoside triphosphates, that can be employed for synthesis of the RNA transcripts in the methods of the invention are provided in the amount of from preferably about 0.25 to about 6 mM, more preferably about 0.5 to about 5 mM, even more preferably about 0.75 to about 4 mM, and most preferably about 1 to about 3 mM.

The oligonucleotide components of the amplification reactions of the invention are generally in excess of the number of target nucleic acid sequence to

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be amplified. They can be provided at about or at least about any of the following: 10, 10^2, 10^4, 10^6, 10^8, 10^{10}, 10^{12} times the amount of target nucleic acid. Primers and PTO can each be provided at about or at least about any of the following concentrations: 50 nM, 100 nM, 500 nM, 1000 nM, 2500 nM, 5000 nM.

In one embodiment, the foregoing components are added simultaneously at the initiation of the amplification process. In another embodiment, components are added in any order prior to or after appropriate timepoints during the amplification process, as required and/or permitted by the amplification reaction. Such timepoints, some of which are noted below, can be readily identified by a person of skill in the art. The enzymes used for nucleic acid amplification according to the methods of the invention can be added to the reaction mixture either prior to the target nucleic acid denaturation step, following the denaturation step, or following hybridization of the primer to the target RNA, as determined by their thermal stability and/or other considerations known to the person of skill in the art. The first strand cDNA (first primer extension product) and the second strand cDNA (second primer extension product) synthesis reactions can be performed consecutively, followed by the amplification steps (for example, binding of propromoter polynucleotide and transcription). In these embodiments, the reaction conditions and components may be varied between the different reactions.

The amplification reactions can be stopped at various timepoints, and resumed at a later time. Said timepoints can be readily identified by a person of skill in the art. One timepoint is at the end of first strand cDNA synthesis. Another timepoint is at the end of second strand cDNA synthesis. Methods for stopping the reactions are known in the art, including, for example, cooling the reaction mixture to a temperature that inhibits enzyme activity or heating the reaction mixture to a temperature that destroys an enzyme. Methods for resuming the reactions are also known in the art, including, for example, raising the temperature of the reaction mixture to a temperature that permits enzyme activity or replenishing a destroyed (depleted) enzyme. In some embodiments, one or more of the components of the reactions is replenished prior to, at, or following

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the resumption of the reactions. Alternatively, the reaction can be allowed to proceed (i.e., from start to finish) without interruption.

The reaction can be allowed to proceed without purification of intermediate complexes, for example, to remove primer. Products can be purified at various timepoints, which can be readily identified by a person of skill in the art. One timepoint is at the end of first strand cDNA synthesis. Another timepoint is at the end of second strand cDNA synthesis.

The detection of the amplification product is indicative of the presence of the target sequence. Quantitative analysis is also feasible. Direct and indirect detection methods (including quantitation) are well known in the art. For example, by comparing the amount of product amplified from a test sample containing an unknown amount of a polynucleotide containing a target sequence to the product of amplification of a reference sample that has a known quantity of a polynucleotide that contains the target sequence, the amount of target sequence in the test sample can be determined. The amplification methods of the invention can also be extended to analysis of sequence alterations and sequencing of the target nucleic acid. Further, detection could be effected by, for example, examination of translation products from RNA amplification products.

Compositions and kits of the invention

The invention also provides compositions and kits used in the methods described herein. The compositions may be any component(s), reaction mixture and/or intermediate described herein, as well as any combination thereof.

For example, the invention provides compositions comprising: (a) a first primer; (b) a second primer (which can be a random primer); and (c) a propromoter polynucleotide (such as a PTO). In some embodiments, the compositions further comprises: (d) a third primer (which can be a random primer). In some embodiments, the second primer and/or third primer is a random primer. In some embodiments, the propromoter polynucleotide ((c), above) is capable of hybridizing to the complement of the 5' portion of the first primer.

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The invention also provides compositions comprising a propromoter polynucleotide (such as a PTO) capable of hybridizing to a 3' portion of a second primer extension that is complement of a 5' portion of a first primer used to create first primer extension product.

The invention also provides compositions comprising (a) a first primer; (b) a second primer (which can be a random primer); and (c) a composite primer, wherein the composite primer comprises a 5' RNA portion and a DNA portion. In some embodiments, the composition further comprises one or more of the following: DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, and an agent (generally an enzyme) that cleaves RNA from an RNA/DNA heteroduplex.

The invention also provides compositions comprising a propromoter polynucleotide, wherein the propromoter is hybridizable to a sequence in the second primer extension product comprising the complement of the 5' portion of a first prime, wherein the first primer is extended to form the first primer extension product.

In another aspect, the invention provides complexes and/or reaction intermediates produced (present) in any of the methods described herein. Examples of such complexes are schematically depicted in Figures 1,2 and 3. In one example, a complex of the invention is a complex comprising: (a) a second or third primer extension product; and (b) a propromoter polynucleotide (for example, a PTO).In yet another example, the invention provides compositions comprising a complex of (a) a third primer extension product; and (b) a propromoter polynucleotide (which can be a PTO). In yet another example, the invention provides compositions comprising a complex of (a) a second primer extension product, generated by denaturation of a hybridized first and second primer extension product; and (b) a composite primer hybridizable to the second primer extension product.

The invention also provides compositions comprising the amplification products described herein. Accordingly, the invention provides a population of anti-sense RNA molecules which are copies of a target sequence, which are produced by any of the methods described herein. The invention also provides a

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population of anti-sense polynucleotides (generally DNA) molecules, which are produced by any of the methods described herein.

The compositions are generally in a suitable medium, although they can be in lyophilized form. Suitable media include, but are not limited to, aqueous media (such as pure water or buffers).

The invention provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any one or more of the uses described herein, and, accordingly, may contain instructions for any one or more of the following uses: amplifying a RNA sequence of interest, sequencing, genotyping (nucleic acid mutation detection), preparation of an immobilized nucleic acid (which can be a nucleic acid immobilized on a microarray), characterizing nucleic acids using the amplified nucleic acid products generated by the methods of the invention; gene expression profiling, subtractive hybridization; preparation of probes for subtractive hybridization; and preparing libraries (which can be cDNA and/or differential hybridization libraries).

The kits of the invention comprise one or more containers comprising any combination of the components described herein, and the following are examples of such kits. For example, the invention provides kits that comprise a first primer that comprises a sequence that when introduced into the amplification steps of the methods of the invention results in generation of an intermediate polynucleotide to which a propromoter polynucleotide can hybridize. The invention also provides kits that further comprise a second primer and/or a third primer, either of both of which can be a random primer. The kits can contain further components, such as any of (a) a propromoter polynucleotide (such as a PTO); and (b) any of the enzymes described herein, such as an enzyme which cleaves RNA from an RNA/DNA hybrid (for example, RNaseH), a DNA polymerase (RNA-dependent or DNA-dependent) or an RNA polymerase. With respect to compositions containing a random primer, these compositions may also contain a plurality of random primers (i.e., a population of random primers having different sequences).

Kits may also optionally include any of one or more of the enzymes described herein, as well as deoxynucleoside triphosphates and/or ribonucleoside

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triphosphates. Kits may also include one or more suitable buffers (as described herein). Kits useful for nucleic acid sequencing may optionally include labeled or unlabeled nucleotide analogs that upon incorporation into a primer extension product effect termination of nucleotide polymerization. One or more 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 any of the methods described herein. Each component can be packaged in separate containers or some components can be combined in one container where cross-reactivity and shelf life permit.

The kits of the invention may optionally include a set of instructions, generally written instructions, although electronic storage media (*e.g.*, magnetic diskette or optical disk) containing instructions are also acceptable, relating to the use of components of the methods of the invention for the intended nucleic acid amplification, and/or, as appropriate, for using the amplification products for purposes such as nucleic acid sequencing and detection of sequence mutation. The instructions included with the kit generally include information as to reagents (whether included or not in the kit) necessary for practicing the methods of the invention, instructions on how to use the kit, and/or appropriate reaction conditions. For example, the invention provides kits that comprise a first primer that comprises a sequence the complement of which is hybridizable by a propromoter polynucleotide, and instructions for using the primer to amplify RNA. In another example, kits can further comprise a second primer and/or a third primer, and optionally instructions for using the primers to amplify RNA. In other examples, the kits can contain further components, such as any of (a) a propromoter polynucleotide (such as a PTO); and (b) any of the enzymes described herein, such as an enzyme which cleaves RNA from an RNA/DNA hybrid (for example, RNaseH), DNA polymerase (RNA-dependent or DNA-dependent) and RNA polymerase. In another example, a kit comprises (a) a composite primer; and (b) instructions for amplifying RNA according to any of the methods described herein. In some embodiments, said kit further comprises a PTO. In other embodiments, said kit further comprises one or more of the following components: (a) a first primer; (b) a second primer; (c) an agent

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(generally RNase H) capable of cleaving RNA from RNA/DNA heteroduplexes; DNA-dependent DNA polymerase; and RNA-dependent DNA-polymerase. Any of these kits can further comprise instructions for using the components to amplify RNA.

The component(s) of the kit may be packaged in any convenient, appropriate packaging. The components may be packaged separately, or in one or multiple combinations. Where kits are provided for practicing amplification methods of the invention, the RNA polymerase (if included) is preferably provided separately from the components used in the steps prior to the transcription steps.

The relative amounts of the various components in the kits can be varied widely to provide for concentrations of the reagents that substantially optimize the reactions that need to occur to practice the methods disclosed herein and/or to further optimize the sensitivity of any assay.

The invention also provides systems for effecting the methods described herein. These systems comprise various combinations of the components discussed above. For example, the invention provides systems for amplifying a target RNA, comprising: (a) a first primer; (b) a second primer (which can be a random primer); (c) an RNA-dependent DNA polymerase; (d) a DNA-dependent DNA polymerase; (e) a propromoter polynucleotide; and (f) an enzyme that cleaves RNA from an RNA/DNA hybrid. The system may further comprise: (g) a third primer (which can be a random primer). The system may also further comprise a composite primer that hybridizes to a second strand cDNA. A system generally includes one or more apparatuses for performing the amplification methods of the invention. Such apparatuses include, for example, heating devices (such as heating blocks or water baths) and apparatuses which effect automation of one or more steps of the methods described herein.

The invention also provides reaction mixtures (or compositions comprising reaction mixtures) which contain various combinations of components described herein. An example of a reaction mixture is (a) a complex of a first primer extension product and a second primer extension product; (b) a polynucleotide comprising a propromoter sequence (for example, a PTO); and (c)

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RNA polymerase. Other reaction mixtures are described herein and are encompassed by the invention.

Methods using the amplification methods and compositions of the invention

The methods and compositions of the invention can be used for a variety of purposes. For purposes of illustration, methods of sequencing, genotyping (nucleic acid mutation detection), preparation of an immobilized nucleic acid (which can be a nucleic acid immobilized on a microarray), and characterizing nucleic acids using the amplified nucleic acid products generated by the methods of the invention, are described. Methods of expression profiling, methods of subtractive hybridization and the preparation of probes for subtractive hybridization, and methods of preparing libraries (which can be cDNA and/or differential hybridization libraries) are also described.

Sequencing of RNA targets using the methods of the invention

The amplification methods of the invention are useful, for example, for sequencing of an RNA sequence of interest. The sequencing process is carried out as described for the amplification methods described herein.

The amplification methods of the invention are useful, for example, for sequencing of an RNA sequence of interest. The sequencing process is carried out by amplifying a target RNA containing the sequence of interest by any of the methods described herein. Addition of nucleotides during primer extension is analyzed using methods known in the art, for example, incorporation of a terminator nucleotide or sequencing by synthesis (*e.g.* pyrosequencing).

In embodiments wherein the end product is in the form of displaced DNA primer extension products, in addition to the nucleotides, such as natural deoxyribonucleotide triphosphates (dNTPs), that are used in the amplification methods, appropriate nucleotide triphosphate analogs, which may be labeled or unlabeled, that upon incorporation into a primer extension product effect termination of primer extension, may be added to the reaction mixture.

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Preferably, the dNTP analogs are added after a sufficient amount of reaction time has elapsed since the initiation of the amplification reaction such that a desired amount of second primer extension product or fragment extension product has been generated. Said amount of the time can be determined empirically by one skilled in the art.

In embodiments wherein the end product is in the form of RNA products, sequencing can be based on premature (deliberate) termination of RNA transcription. The inclusion of rNTP analogs, which may be labeled or unlabeled, that upon incorporation into an RNA transcript effects termination of rNTP polymerization in the reaction mixture, will result in production of truncated RNA products, which result from blocking of the RNA polymerase at sites of incorporation of the analogs.

Suitable analogs (dNTP and rNTP) include those commonly used in other sequencing methods and are well known in the art. Examples of dNTP analogs include dideoxyribonucleotides. Examples of rNTP analogs (such as RNA polymerase terminators) include 3'-dNTP. Sasaki et al., Biochemistry (1998) 95:3455-3460. These analogs may be labeled, for example, with fluorochromes or radioisotopes. The labels may also be labels which are suitable for mass spectroscopy. The label may also be a small molecule which is a member of a specific binding pair, and can be detected following binding of the other member of the specific binding pair, such as biotin and streptavidin, respectively, with the last member of the binding pair conjugated to an enzyme that catalyzes the generation of a detectable signal that could be detected by methods such as colorimetry, fluorometry or chemiluminescence. All of the above examples are well known in the art. These are incorporated into the primer extension product or RNA transcripts by the polymerase and serve to stop further extension along a template sequence. The resulting truncated polymerization products are labeled. The accumulated truncated products vary in length, according to the site of incorporation of each of the analogs, which represent the various sequence locations of a complementary nucleotide on the template sequence.

Analysis of the reaction products for elucidation of sequence information can be carried out using any of various methods known in the art. Such methods

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include gel electrophoresis and detection of the labeled bands using appropriate scanner, sequencing gel electrophoresis and detection of the radiolabeled band directly by phosphorescence such as Molecular Dynamics reader, capillary electrophoresis adapted with a detector specific for the labels used in the reaction, and the like. The label can also be a ligand for a binding protein which is used for detection of the label in combination with an enzyme conjugated to the binding protein, such as biotin-labeled chain terminator and streptavidin conjugated to an enzyme. The label is detected by the enzymatic activity of the enzyme, which generates a detectable signal. As with other sequencing methods known in the art, the sequencing reactions for the various nucleotide types (A, C, G, T or U) are carried out either in a single reaction vessel, or in separate reaction vessels (each representing one of the various nucleotide types). The choice of method to be used is dependent on practical considerations readily apparent to one skilled in the art, such as the nucleotide tri phosphate analogs and/or label used. Thus, for example, when each of the analogs is differentially labeled, the sequencing reaction can be carried out in a single vessel. The considerations for choice of reagent and reaction conditions for optimal performance of sequencing analysis according to the methods of the invention are similar to those for other previously described sequencing methods. The reagent and reaction conditions should be as described above for the nucleic acid amplification methods of the invention.

Mutation detection, including mutation detection based on single stranded conformation polymorphism utilizing the amplification methods of the invention

The polynucleotide (generally, RNA and DNA) amplification products generated according to the methods of the invention are also suitable for analysis for the detection of any alteration in the target nucleic acid sequence, as compared to a reference nucleic acid sequence which is identical to the target nucleic acid sequence other than the sequence alteration. The sequence alterations may be sequence alterations present in the genomic sequence or may be sequence alterations which are not reflected in the genomic DNA sequences, for example,

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alterations due to post transcriptional alterations, and/or mRNA processing, including splice variants.

The RNA and DNA products of the amplification methods are suitable for single stranded conformation polymorphism (rSSCP) based mutation detection. The amplification methods of the invention can be directly linked to appropriate means for detecting single stranded conformation polymorphism, such as an electrophoretic separation method for the identification of specific mobility pattern of the single stranded RNA or DNA products for the elucidation of the presence of specific sequence feature(s), and/or the presence of any difference in a test nucleic acid as compared to a reference nucleic acid.

Methods based on gel electrophoresis or capillary electrophoresis can be used for the detection and analysis of the various single stranded conformational isomers. Alternatively, it is also likely that cleavage of the single stranded RNA product using nucleases which recognize sequence dependent secondary structures may be useful for the determination of sequence specific conformation polymorphism. Such nucleases are known in the art, such as the Cleavase assay (Third Wave). The electrophoretic methods are potentially more suitable for high throughput mutation, or genotyping, detection methods.

The determination of sequence specific electrophoretic pattern for a given nucleic acid sequence is useful for, for example, the detection of specific alleles of a test sequence. Furthermore, it is expected that an electrophoretic mobility pattern for the various alleles could be well differentiated, thus allowing the detection of two alleles in a nucleic acid sample from a single individual, as required for heterozygous genotype, or multiple alleles. Any alteration in the test nucleic acid sequence, such as base substitution, insertions or deletion, could be detected using this method. The method is expected to be useful for detection of specific single base polymorphism, SNP, and the discovery of new SNPs. Thus, the invention also provides methods for detecting a polynucleotide comprising a single nucleotide polymorphism, comprising: (a) amplifying a target polynucleotide using any of the methods described herein; and (b) analyzing the amplification products for single stranded conformation, wherein a difference in conformation as compared to a reference single stranded polynucleotide indicates

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a single nucleotide polymorphism in the target polynucleotide, whereby a polynucleotide comprising a single nucleotide polymorphism is detected.

Other art recognized methods of analysis for the detection of any alteration in the target nucleic acid sequence, as compared to a reference nucleic acid sequence, are suitable for use with the single stranded nucleic acid products of the amplification methods of the invention. Such methods are well-known in the art, and include various methods for the detection of specific defined sequences including methods based on allele specific primer extension, allele specific probe ligation, differential probe hybridization, and limited primer extension. See, for example, Kurn et al, U.S. Patent No. 6,251,639 B1; U.S. Patent Nos. 5,888,819; 6,004,744; 5,882,867; 5, 854, 033; 5,710,028; 6,027,889; 6,004,745; 5,763,178; 5,011,769; 5,185,243; 4,876,187; 5,882,867; 5,731,146; WO US88/02746; WO 99/55912; WO92/15712; WO 00/09745; WO 97/32040; WO 00/56925; and 5,660,988. Thus, the invention also provides methods for detection of a mutation in an RNA sequence of interest comprising a single nucleotide polymorphism, comprising: (a) amplifying a target RNA using any of the methods described herein; and (b) analyzing the amplification products for presence of an alteration (mutation) as compared to a reference single stranded polynucleotide.

Method of immobilizing single stranded nucleic acids

The single stranded polynucleotide (generally RNA and DNA) products of some of the amplification methods of the invention are suitable for immobilizing to a surface. The single stranded products are particularly suitable for preparing microarrays comprising the single stranded amplification products.

Amplification products can be immobilized and/or attached to a solid or semi-solid support or surface, which may be made, e.g., from glass, plastic (e.g., polypropylene, nylon), polyacrylamide, nitrocellulose, or other materials.

Several techniques are well-known in the art for immobilizing nucleic acids to a solid substrate such as a glass slide. One method is to incorporate modified bases or analogs that contain a moiety that is capable of attachment to a

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solid substrate, such as an amine group, a derivative of an amine group or another group with a positive charge, into the amplified nucleic acids. The amplified product is then contacted with a solid substrate, such as a glass slide, which is coated with an aldehyde or another reactive group which will form a covalent link with the reactive group that is on the amplified product and become covalently attached to the glass slide. Microarrays comprising the amplified products can be fabricated using a Biodot (BioDot, Inc. Irvine, CA) spotting apparatus and aldehyde-coated glass slides (CEL Associates, Houston, TX). Amplification products can be spotted onto the aldehyde-coated slides, and processed according to published procedures (Schena et al., *Proc. Natl. Acad. Sci. U.S.A.* (1996), 93: 10614-10619). Arrays can also be printed by robotics onto glass, nylon (Ramsay, G., *Nature Biotechnol.* (1998), 16:40-44), polypropylene (Matson, et al., *Anal. Biochem.* (1995), 224(1):110-6), and silicone slides (Marshall, A. and Hodgson, J., *Nature Biotechnol.* (1998), 16:27-31). Other approaches to array assembly include fine micropipetting within electric fields (Marshall and Hodgson, *supra*), and spotting the polynucleotides directly onto positively coated plates. Methods such as those using amino propyl silicon surface chemistry are also known in the art, as disclosed at http://www.cmt.corning.com and http://cmgm.stanford.edu/pbrown/.

One method for making microarrays is by making high-density polynucleotide arrays. Techniques are known for rapid deposition of polynucleotides (Blanchard et al., *Biosensors & Bioelectronics*, 11:687-690). Other methods for making microarrays, e.g., by masking (Maskos and Southern, *Nuc. Acids. Res.* (1992),20:1679-1684), may also be used. In principle, and as noted above, any type of array, for example, dot blots on a nylon hybridization membrane, could be used. However, as will be recognized by those skilled in the art, very small arrays will frequently be preferred because hybridization volumes will be smaller.

The amplified polynucleotides may be spotted as a matrix on substrates comprising paper, glass, plastic, polypropylene, nylon, polyacrylamide, nitrocellulose, silicon, optical fiber or any other suitable solid or semi-solid (e.g.,

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thin layer of polyacrylamide gel (Khrapko, et al., *DNA Sequence* (1991), 1:375-388) surface.

An array may be assembled as a two-dimensional matrix on a planar substrate or may have a three-dimensional configuration comprising pins, rods, fibers, tapes, threads, beads, particles, microtiter wells, capillaries, cylinders and any other arrangement suitable for hybridization and detection of target molecules. In one embodiment the substrate to which the amplification products are attached is magnetic beads or particles. In another embodiment, the solid substrate comprises an optical fiber. In yet another embodiment, the amplification products are dispersed in fluid phase within a capillary which, in turn, is immobilized with respect to a solid phase.

Characterization of nucleic acids

The methods of the invention are particularly amenable to quantitative analysis, as sufficient single stranded polynucleotide (generally, DNA and RNA) products are produced which accurately reflect the representation of the various mRNA in the starting material. The amplified products can be analyzed using, for example, probe hybridization techniques known in the art, such as Northern blotting, and hybridizing to probe arrays. In addition, the single stranded polynucleotide products may serve as starting material for other starting material for other analytical and/or quantification methods known in the art, such as real time PCR, quantitative TaqMan, quantitative PCR using molecular beacons, methods described in Kurn, U.S. Patent No. 6,251,639, etc. Thus, the invention includes those further analytical and/or quantification methods as applied to any of the products of the methods herein.

In another embodiment, the amplification methods of the invention are utilized to generate multiple copies of single stranded polynucleotide (DNA or RNA) products that are labeled by the incorporation of labeled nucleotides during DNA or RNA polymerization. For example, amplification according to the methods of the invention can be carried out with suitable labeled dNTPs or rNTPs. These labeled nucleotides can be directly attached to a label, or can

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comprise a moiety which could be attached to a label. The label may be attached covalently or non-covalently to the amplification products. Suitable labels are known in the art, and include, for example, a ligand which is a member of a specific binding pair which can be detected/quantified using a detectable second member of the binding pair. Thus, amplification of total mRNA according to the methods of the invention in the presence of, for example, Cy3-dUTP or Cy5-dUTP results in the incorporation of these nucleotides into the amplification products.

The labeled amplified products are particularly suitable for analysis (for example, detection and/or quantification) by contacting them with, for example, microarrays (of any suitable surface, which includes glass, chips, plastic), beads, or particles, that comprise suitable probes such as cDNA and/or oligonucleotide probes. Thus, the invention provides methods to characterize (for example, detect and/or quantify) an RNA sequence of interest by generating labeled polynucleotide (generally, DNA or RNA) products using amplification methods of the invention, and analyzing the labeled products. Analysis of labeled products can be performed by, for example, hybridization of the labeled amplification products to, for example, probes immobilized at, for example, specific locations on a solid or semi-solid substrate, probes immobilized on defined particles, or probes immobilized on blots (such as a membrane), for example arrays, which have been described above. Other methods of analyzing labeled products are known in the art, such as, for example, by contacting them with a solution comprising probes, followed by extraction of complexes comprising the labeled amplification products and probes from solution. The identity of the probes provides characterization of the sequence identity of the amplified products, and thus by extrapolation the identity of the target RNA present in a sample. Hybridization of the labeled products is detectable, and the amount of specific labels that are detected is proportional to the amount of the labeled amplification products of a specific RNA sequence of interest. This measurement is useful for, for example, measuring the relative amounts of the various RNA species in a sample, which are related to the relative levels of gene expression, as described herein. The amount of labeled products (as indicated by, for example, detectable

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signal associated with the label) hybridized at defined locations on an array can be indicative of the detection and/or quantification of the corresponding target RNA species in the sample.

The labeled amplified products are particularly suitable for analysis (for example, detection and/or quantification and/or determining presence or absence of) by contacting them with, for example, microarrays that comprise suitable probes such as cDNA and/or oligonucleotide probes. Thus, the invention provides methods to characterize (for example, detect and/or quantify and/or determine presence or absence of) an RNA sequence of interest by generating labeled polynucleotide (generally, RNA or DNA) products using amplification methods of the invention, and analyzing the labeled products. Analysis of labeled products can be performed by, for example, hybridization of the labeled amplification products to, for example, probes immobilized at, for example, specific locations on a solid or semi-solid substrate, probes immobilized on defined particles, or probes immobilized on blots (such as a membrane), for example arrays, which have been described above. Other methods of analyzing labeled products are known in the art, such as, for example, by contacting them with a solution comprising probes, followed by extraction of complexes comprising the labeled amplification products and probes from solution. The identity of the probes provides characterization of the sequence identity of the amplified products, and thus by extrapolation the identity of the target RNA present in a sample. Hybridization of the labeled products is detectable, and the amount of specific labels that are detected is proportional to the amount of the labeled amplification products of a specific RNA sequence of interest. This measurement is useful for, for example, measuring the relative amounts of the various RNA species in a sample, which are related to the relative levels of gene expression. The amount of labeled products (as indicated by, for example, detectable signal associated with the label) hybridized at defined locations on an array can be indicative of the detection and/or quantification and/or presence or absence of the corresponding target RNA species in the sample.

Determination of gene expression profile

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The amplification methods of the invention are particularly suitable for use in determining the levels of expression of multiple genes in a sample since the methods described herein are capable of amplifying multiple target RNAs in the same sample. As described above, amplification products can be detected and quantified by various methods, as described herein and/or known in the art. Since RNA is a product of gene expression, the levels of the various RNA species, such as mRNAs, in a sample is indicative of the relative expression levels of the various genes (gene expression profile). Thus, determination of the amount of RNA sequences of interest present in a sample, as determined by quantifying amplification products of the sequences, provides for determination of the gene expression profile of the sample source.

Accordingly, the invention provides methods of determining gene expression profile in a sample, said method comprising: amplifying single stranded product from at least one RNA sequence of interest in the sample, using any of the methods described herein ; and determining amount of amplification products of each RNA sequence of interest, wherein each said amount is indicative of amount of each RNA sequence of interest in the sample, whereby the expression profile in the sample is determined. Generally, labeled products are generated. In one embodiment, the target RNA is mRNA. It is understood that amount of amplification product may be determined using quantitative and/or qualitative methods. Determining amount of amplification product includes determining whether amplification product is present or absent. Thus, an expression profile can includes information about presence or absence of one or more RNA sequence of interest. "Absent" or "absence" of product, and "lack of detection of product" as used herein includes insignificant, or *de minimus* levels.

The methods of expression profiling are useful in a wide variety of molecular diagnostic, and especially in the study of gene expression in essentially any mammalian cell (including a single cell) or cell population. A cell or cell population (e.g. a tissue) may be from, for example, blood, brain, spleen, bone, heart, vascular, lung , kidney, pituitary, endocrine gland, embryonic cells, tumors, or the like. Expression profiling is also useful for comparing a control (normal)

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sample to a test sample, including test samples collected at different times, including before , after, and/or during development, a treatment, and the like.

Method of preparing a library

The single stranded polynucleotides (generally DNA and RNA) products of the methods of the invention are useful in preparing libraries, including cDNA libraries and subtractive hybridization libraries. Using the methods of the invention, libraries may be prepared from limited amount of starting material, for example, mRNA extracted from limited amount of tissue or even single cells. Accordingly, in one aspect, the methods of the invention provides preparing a library from the single stranded DNA or RNA products of the invention. In still another aspect, the invention provides methods for making a library, said method comprising: preparing a subtractive hybridization probe using any of the methods described herein.

Accordingly, in one aspect, the methods of the invention provides preparing a library from the single stranded DNA or RNA products of the invention. In some embodiments, the library is a cDNA library.

Methods of subtractive hybridization

The amplification methods of the invention are particularly suitable for use in subtractive hybridization methods, since the methods described herein are capable of amplifying multiple target RNAs in the same sample, and the methods of the invention are suitable for producing large amounts of single stranded anti-sense DNA and RNA product suitable for use as "driver" in subtractive hybridization. For example, two nucleic acid populations, one sense and one antisense, can be allowed to mix together with one population present in molar excess ("driver"). Sequence present in both populations will form hybrids, while sequences present in only one population remain single-stranded. Thereafter, various well known techniques are used to separate the unhybridized molecules representing differentially expressed sequences. See, e.g., Hamson et al., U.S. Patent No. 5,589,339; Van Gelder, U.S. Patent No. 6,291,170;

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Accordingly, the invention provides methods for performing subtractive hybridization, said methods comprising: (a) preparing multiple copies (generally, DNA) of the complement of at least one RNA sequences of interest from a first RNA population using any of the amplification methods described herein; and (b) hybridizing the multiple copies to a second mRNA population, whereby a subpopulation of the second mRNA population forms a complex with a nucleotide DNA copy. The invention also provides methods for performing subtractive hybridization, said methods comprising: hybridizing multiple copies of the complement of at least one RNA sequences of interest from a first RNA population using any of the amplification methods described herein to a second mRNA population, whereby a subpopulation of the second mRNA population forms a complex with a copy. In some embodiments, "driver" single stranded anti-sense DNA product of the methods of the invention is combined with tester (sense) mRNA species. In another aspect, the invention provides methods of differential amplification in which single stranded driver (antisense) DNA sequences that hybridize with tester mRNA sequence are subjected to cleavage by an agent that cleaves RNA present in a DNA/RNA hybrid, such as RNase H. Cleavage of the mRNA results in the inability to generate single stranded DNA product from the test mRNA strands. Conversely, non-cleaved tester (i.e., tester mRNA that did not hybridize to driver DNA molecules) may serve as a substrate for subsequent amplification. Amplified differentially expressed products have many uses, including as a differential expression probe, to produce differential expression libraries Accordingly, in another aspect, the invention provides methods comprising hybridizing multiple polynucleotide (generally, DNA) copies of the complement of at least one RNA sequences of interest from a first RNA population using any of the amplification methods described herein to a second mRNA population, whereby a subpopulation of the second mRNA population forms a complex with a DNA copy; (b) cleaving RNA in the complex of step (a) with an enzyme that cleaves RNA from an RNA/DNA hybrid; and (c) amplifying an unhybridized subpopulation of the second mRNA population, whereby multiple copies of single stranded polynucleotide (generally, DNA)

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complementary to the unhybridized subpopulation of the second mRNA population are generated.

The following Examples are provided to illustrate, but not limit, the invention.

EXAMPLES

Example 1: Amplification of total mRNA

0.1 μg of total mRNA is combined with primer 1 (provided at a concentration of from 0.1 to 1 μM), PTO oligonucleotide (provided at a concentration of from 0.1 to 1 μM), primer 2 (provided at a concentration of from 0.1 to 1 μM) in a total volume of about 10 μl in a buffer containing 40 mM Tris, pH 8.5, 5 mM DTT, 12 mM $MgCl_2$, 70 mM KCl, 108.8 μg/ml BSA; 1 mM of each dNTP, 2 mM each of rATP, rUTP, rCTP, 1.5 mM rGTP, and 0.5 mM rITP). The mixture is incubated at 65^0C for 2 minutes, and cooled down to 37^0C (or 42^0C). 10 μl of an enzyme mixture containing T7 RNA polymerase (40 to 80 U), MMLV reverse transcriptase (10 to 30 U), and RNase H (1 to 3 U), is added to the reaction mixture, and the mixture is further incubated for 0.5 to 3 hours.

Aliquots of the reaction mixture are analyzed by gel electrophoresis (5 to 20% PAGE, Novex) for generation of amplification products.

Primer 1 sequence:

GACGGATGCGGTCTTTTTTTTN

"N" denotes a degenerate nucleotide (i.e., it can be A, T, C or G).

Primer 2:

Random hexamer

PTO:

ggAATTCTAATACgACTCACTATAgggAgAgC**GACGGATGCGGTCT**-biotin

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wherein bold letters denote the sequence complementary to the 3'-end of the second primer extension product.

Primer 3:

Primer 3 is the same as primer 1.

Example 2: Amplification of specific mRNA

The experiment of Example 1 is performed, except that primer 2 is substituted with a primer specific for a sequence of a defined mRNA species to be amplified. For example, the amplification of a sequence of GAPDH mRNA is carried out with a primer that hybridizes to a site on the first cDNA strand (first primer extension product) generated from GAPDH mRNA.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the descriptions and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

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CLAIMS

What is claimed is:

1. A method of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising the steps of:

(a) extending a first primer hybridized to a target RNA with an RNA-dependent DNA polymerase, whereby a complex comprising a first primer extension product and the target RNA is produced;

(b) cleaving RNA in the complex of step (b) with an enzyme that cleaves RNA from an RNA/DNA hybrid;

(c) extending a second primer hybridized to the first primer extension product with a DNA-dependent DNA polymerase, whereby a complex comprising the first primer extension product and a second primer extension product is produced;

(d) denaturing the complex of step (c); and

(e) hybridizing to the second primer extension product a propromoter polynucleotide comprising a propromoter and a region which hybridizes to the second primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the target RNA;

whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

2. A method of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising the steps of:

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(a) extending a first primer hybridized to a target RNA with an RNA-dependent DNA polymerase, whereby a complex comprising a first primer extension product and the target RNA is produced;

(b) cleaving RNA in the complex of step (a) with an enzyme that cleaves RNA from an RNA/DNA hybrid;

(c) extending a second primer hybridized to the first primer extension product with a DNA-dependent DNA polymerase, whereby a complex comprising the first primer extension product and a second primer extension product is produced;

(d) denaturing the complex of step (c);

(e) hybridizing to the second primer extension product a propromoter polynucleotide comprising a propromoter and a region which hybridizes to the second primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the target RNA;

(f) extending a third primer hybridized to said RNA transcripts with an RNA-dependent DNA polymerase, whereby a complex comprising a third primer extension product and an RNA transcript is produced;

(g) cleaving RNA in the complex of step (f) with an enzyme that cleaves RNA from an RNA/DNA hybrid;

(h) hybridizing a propromoter polynucleotide comprising a propromoter and a region which hybridizes to a single stranded third primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the target RNA;

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(i) optionally repeating steps (f) to (h);

whereby multiple copies of the complementary sequence of the RNA sequence of interest are produced.

3. The method of claim 1 or 2, wherein the first primer comprises a 5' portion that is not hybridizable to the target RNA.

4. The method of claim 3, wherein said 5' portion comprises a sequence the complement of which is hybridizable by the propromoter polynucleotide in step (f).

5. The method of claim 1 or 2, wherein the target RNA is mRNA.

6. The method of claim 1 or 2, wherein the first primer comprises a poly-T sequence.

7. The method of claim 2, wherein the first primer comprises a 5' portion that is not hybridizable to the target RNA, and wherein said 5' portion comprises a sequence the complement of which is hybridizable by the propromoter polynucleotide in step (g).

8. The method of claim 2, wherein the second primer and the third primer are the same.

9. The method of claim 2, wherein the second primer and the third primer are different.

10. The method of claim 2, wherein the second primer and the third primer hybridize to different complementary sequences.

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11. The method of claim 1 or 2, wherein at least one type of rNTP used is a labeled rNTP, whereby labeled products are generated.

12. The method of claim 1 or 2, wherein the first primer is a random primer.

13. The method of claim 1 or 2, wherein the second primer comprises DNA.

14. The method of claim 2 or two, wherein the second primer comprises a fragment of the target RNA hybridized to the primer extension product, said fragment generated by cleaving RNA in the complex of step (b) with an enzyme that cleaves RNA from an RNA/DNA hybrid.

15. The method of claims 1 or 2, wherein said method comprises generating multiple copies of two or more different sequences of interest.

16. The method of claim 15, wherein said method comprises at least two different first primers.

17. A method of generating multiple copies of the complementary sequence of an RNA sequence of interest comprising incubating a reaction mixture, said reaction mixture comprising::

(a) a single stranded second primer extension product resulting from step (d) of claim 1;

(b) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded second primer extension product; and

an RNA polymerase;

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wherein the incubation is under conditions that permit propromoter polynucleotide hybridization and RNA transcription, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

18. A method of generating multiple copies of the complementary sequence of an RNA sequence of interest comprising incubating a reaction mixture, said reaction mixture comprising:

(a) a single stranded second primer extension product resulting from step (d) of claim 1;

(b) a third primer comprising a sequence hybridizable to an RNA transcript comprising a sequence complementary to the target RNA;

(c) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded second primer extension product;

(d) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded third primer extension product;

(e) an enzyme that cleaves RNA from an RNA/DNA hybrid; and

(f) an RNA polymerase;

wherein the incubation is under conditions that permit primer extension, RNA cleavage, propromoter polynucleotide hybridization and RNA transcription, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

19. A method of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising incubating a reaction mixture, said reaction mixture comprising:

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(a) an RNA transcript from step (e) of claim 1;

(b) a third primer comprising a sequence hybridizable to the RNA transcript;

(c) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded third primer extension product;

(d) an enzyme that cleaves RNA from an RNA/DNA hybrid; and

(e) an RNA polymerase;

wherein the incubation is under conditions that permit primer extension, RNA cleavage, propromoter polynucleotide hybridization and RNA transcription, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

20. A method of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising incubating a reaction mixture, said reaction mixture comprising:

(a) a target RNA;

(b) a first primer comprising a sequence that is hybridizable to the target RNA;

(c) a second primer comprising a sequence hybridizable to an extension product of the first primer;

(d) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded second primer extension product;

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(e) an RNA-dependent DNA polymerase;

(f) a DNA-dependent DNA polymerase;

(g) an RNA polymerase; and

(h) an enzyme that cleaves RNA from an RNA/DNA hybrid;

wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage, propromoter polynucleotide hybridization, and RNA transcription, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

21. A method of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising incubating a reaction mixture, said reaction mixture comprising:

(a) a target RNA;

(b) a first primer comprising a sequence that is hybridizable to the target RNA;

(c) a second primer comprising a sequence hybridizable to an extension product of the first primer;

(d) a third primer comprising a sequence hybridizable to an RNA transcript comprising a sequence complementary to the target RNA;

(e) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded second primer extension product;

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(f) a propromoter polynucleotide comprising a propromoter and a region which is hybridizable to a single stranded third primer extension product;

(g) an RNA-dependent DNA polymerase;

(h) a DNA-dependent DNA polymerase;

(i) an RNA polymerase; and

(j) an enzyme that cleaves RNA from an RNA/DNA hybrid;

wherein the incubation is conditions that permit primer hybridization, primer extension, RNA cleavage, propromoter polynucleotide hybridization, and RNA transcription, whereby multiple copies of the complementary sequence of the RNA sequence of interest are generated.

22. The method of claim 20 or 21, wherein the first primer comprises a 5' sequence that is not hybridizable to the target RNA.

23. The method of claim 22, wherein the 5' sequence comprises a sequence the complement of which is hybridizable by a propromoter polynucleotide.

24. The method of claim 20 or 21, wherein (a) the first primer comprises a poly-T sequence; and (b) the target RNA is mRNA.

25. The method of claim 21, wherein the second primer and the third primer are the same.

26. The method of claim 21, wherein the second primer and the third primer are different.

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27. The method of claim 21, wherein the second primer and the third primer hybridize to different complementary sequences.

28. The method of any of claims 1, 2, 18, 19, 20 and 21, wherein the enzyme that cleaves RNA is RNase H.

29. The method of any of claims 1, 2, 20 and 21, wherein the RNA - dependent DNA polymerase and DNA-dependent DNA polymerase are one enzyme.

30. The method of any of claims 1, 2, 20 and 21, wherein the RNA-dependent DNA polymerase and enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

31. The method of any of claims 1, 2, 20 and 21, wherein the DNA-dependent DNA polymerase and enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

32. The method of any of claims 1, 2, 20 and 21, wherein the DNA-dependent DNA polymerase, the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

33. The method of claim 1 or 20, wherein the propromoter polynucleotide comprises a region at the 3' end which hybridizes to the second primer extension product, whereby DNA polymerase extension of the second primer extension product produces a double stranded promoter from which transcription occurs.

34. The method of claim 33, wherein the propromoter polynucleotide is a propromoter template oligonucleotide.

35. The method of claim 2 or 21, wherein the propromoter polynucleotide comprises a region at the 3' end which hybridizes to the third primer extension

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product, whereby DNA polymerase extension of the second primer extension product produces a double stranded promoter from which transcription occurs.

36. The method of claim 35, wherein the propromoter polynucleotide is a propromoter template oligonucleotide.

37. The method of claim 20 or 21, wherein at least one type of rNTP used is a labeled rNTP, whereby labeled products are generated.

38. A method of generating multiple copies of the complementary sequence of an RNA sequence of interest, said method comprising:

(a) hybridizing a composite primer to a single stranded second primer extension product resulting from step (d) of claim 1, wherein the composite primer comprises an RNA portion and a 3' DNA portion;

(b) extending the composite primer with a DNA-dependent DNA polymerase, whereby a complex comprising a primer extension product and the second primer extension product is formed;

(c) cleaving RNA in the complex of step (b) with an enzyme that cleaves RNA from an RNA/DNA hybrid, such that another composite primer hybridizes to the second primer extension product and repeats primer extension by strand displacement,

whereby multiple copies of the complement of the RNA sequence of interest are produced.

39. The method of claim 38, wherein the RNA portion of the composite primer is 5' with respect to the 3' DNA portion.

40. The method of claim 39, wherein the 5' RNA portion is adjacent to the 3' DNA portion.

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41. The method of claim 38, wherein the first primer comprises the composite primer sequence.

42. The method of claim 38, wherein the composite primer comprises a portion hybridizable to the complement of the 5' portion of the first primer.

43. The method of claim 38, wherein the target RNA is mRNA.

44. The method of claim 38, wherein said method comprises generating multiple copies of two or more different sequences of interest.

45. The method of claim 38, wherein the enzyme that cleaves RNA from an RNA/DNA hybrid is RNase H.

46. The method of claim 38, wherein the RNA -dependent DNA polymerase and DNA-dependent DNA polymerase are the same enzyme.

47. The method of claim 38, wherein the RNA-dependent DNA polymerase and enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

48. The method of claim 38, wherein the DNA-dependent DNA polymerase and enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

49. The method of claim 38, wherein the DNA-dependent DNA polymerase, the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

50. The method of claim 38, wherein at least one type of dNTP used is a labeled dNTP, whereby labeled products are generated.

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51. A method of sequencing an RNA sequence of interest, said method comprising (a) amplifying a target RNA containing the sequence of interest by the method of claim 1 or 20 in the presence of a mixture of rNTPs and rNTP analogs such that transcription is terminated upon incorporation of an rNTP analog; and (b) analyzing the amplification products to determine sequence.

52. The method of claim 51, wherein the target RNA is mRNA.

53. A method of sequencing an RNA sequence of interest, said method comprising (a) amplifying a target RNA containing the sequence of interest by the method of claim 2 or 21, wherein RNA transcripts generated from the second primer extension product are amplified in the presence of a mixture of rNTPs and rNTP analogs such that transcription is terminated upon incorporation of an rNTP analog; and (b) analyzing the amplification products to determine sequence.

54. The method of claim 53, wherein the target RNA is mRNA.

55. A method of detecting a mutation in a target RNA, comprising (a) amplifying the target RNA by any of the methods of claims 1, 2, 20 and 21; and (b) analyzing the amplification products for the presence of a mutation as compared to a reference nucleotide.

56. The method of claim 55, wherein the target RNA is mRNA.

57. A method of detecting a mutation in a target RNA by single stranded conformation polymorphism, comprising (a) amplifying the target RNA by any of the methods of claims 1, 2, 20 and 21; and (b) analyzing the amplification products for single stranded conformation, wherein a difference in conformation as compared to a reference single stranded polynucleotide indicates a mutation in the target polynucleotide.

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58. The method of claim 57, wherein the target RNA is mRNA.

59. A method of producing a nucleic acid immobilized to a substrate comprising (a) amplifying a target RNA by any of the methods of claims 1, 2, 20 and 21; and (b) immobilizing the amplification products on a substrate.

60. The method of claim 59, wherein the target RNA is mRNA.

61. The method of claim 59, wherein the substrate is a microarray.

62. A method of characterizing an RNA sequence of interest, comprising (a) amplifying a target RNA by the method of claim 10; and (b) analyzing the labeled RNA products.

63. The method of claim 62, wherein step (b) comprises contacting the labeled DNA products with at least one probe.

64. The method of claim 63, wherein the at least one probe is provided as a microarray.

65. The method of claim 64, wherein the microarray comprises at least one probe immobilized on a substrate fabricated from a material selected from the group consisting of paper, ceramic, glass, plastic, polypropylene, polystyrene, nylon, polyacrylamide, nitrocellulose, silicon, and optical fiber.

66. The method of claim 65, wherein the probe is immobilized on the substrate in a two-dimensional configuration or a three-dimensional configuration comprising pins, rods, fibers, tapes, threads, beads, particles, microtiter wells, capillaries, and cylinders.

67. The method of claim 62, wherein the target RNA is mRNA.

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68. The method of claim 62, wherein step (b) of analyzing the labeled RNA products comprises determining amount of said products, whereby the amount of the RNA sequence of interest present in a sample is quantified.

69. A method of characterizing an RNA sequence of interest, comprising (a) amplifying a target RNA by the method of claim 37; and (b) analyzing the labeled RNA products.

70. The method of claim 69, wherein step (b) comprises contacting the labeled DNA products with at least one probe.

71. The method of claim 70, wherein the at least one probe is provided as a microarray.

72. The method of claim 71, wherein the microarray comprises at least one probe immobilized on a substrate fabricated from a material selected from the group consisting of paper, glass, plastic, polypropylene, nylon, polyacrylamide, nitrocellulose, silicon, and optical fiber.

73. The method of claim 72, wherein the probe is immobilized on the substrate in a two-dimensional configuration or a three-dimensional configuration comprising pins, rods, fibers, tapes, threads, beads, particles, microtiter wells, capillaries, and cylinders.

74. The method of claim 69, wherein the target RNA is mRNA.

75. The method of claim 69, wherein step (b) of analyzing the labeled RNA products comprises determining amount of said products, whereby the amount of the RNA sequence of interest present in a sample is quantified.

76. A method of determining gene expression profile in a sample, said method comprising: (a) amplifying at least two RNA sequences of interest in the

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sample using the method of any of claims 1 or 2; and (b) determining amount of amplification products of each RNA sequence of interest, wherein each said amount is indicative of amount of each RNA sequence of interest in the sample, whereby the gene expression profile in the sample is determined.

77. The method of claim 76, wherein each target RNA is mRNA.

78. A method of preparing a library, said method comprising: (a) amplifying at least two RNA sequences of interest using the method of any of claims 1 and 2; and (b) preparing a library from amplified single stranded DNA or RNA product.

79. The method of claim 78, wherein the first primer is a random primer

80. The method of claim 78, wherein the first primer comprises a poly-T portion.

81. A method of performing subtractive hybridization, said method comprising: (a) preparing multiple DNA copies of the complement of one or more RNA sequences of interest from a first RNA population using the methods of claim 38; and (b) hybridizing the multiple copies to a second mRNA population, whereby a subpopulation of the second mRNA population forms a complex with the DNA copies.

82. A method of performing subtractive hybridization, said method comprising: (a) preparing multiple DNA copies of the complement of at least two RNA sequences of interest from a first RNA population using the method of claim 38; (b) hybridizing the multiple copies to a second mRNA population, whereby a subpopulation of the second mRNA population forms a complex with a DNA copy; (c) cleaving RNA in the complex of step (b) with an enzyme that cleaves RNA from an RNA/DNA hybrid; and (d) amplifying the unhybridized subpopulation of the second mRNA population, whereby multiple copies of single

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stranded DNA complementary to the unhybridized subpopulation of the second mRNA population are generated.

83 A kit comprising a first primer that comprises a sequence the complement of which is hybridizable by a propromoter polynucleotide, and instructions for using the primer to amplify RNA using the methods of any of claims 1, 2, 17, 18, 19, 20, 21, and 38.

82. The kit of claim 83, further comprising a second primer.

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FIGURE 1

Exponential isothermal amplification

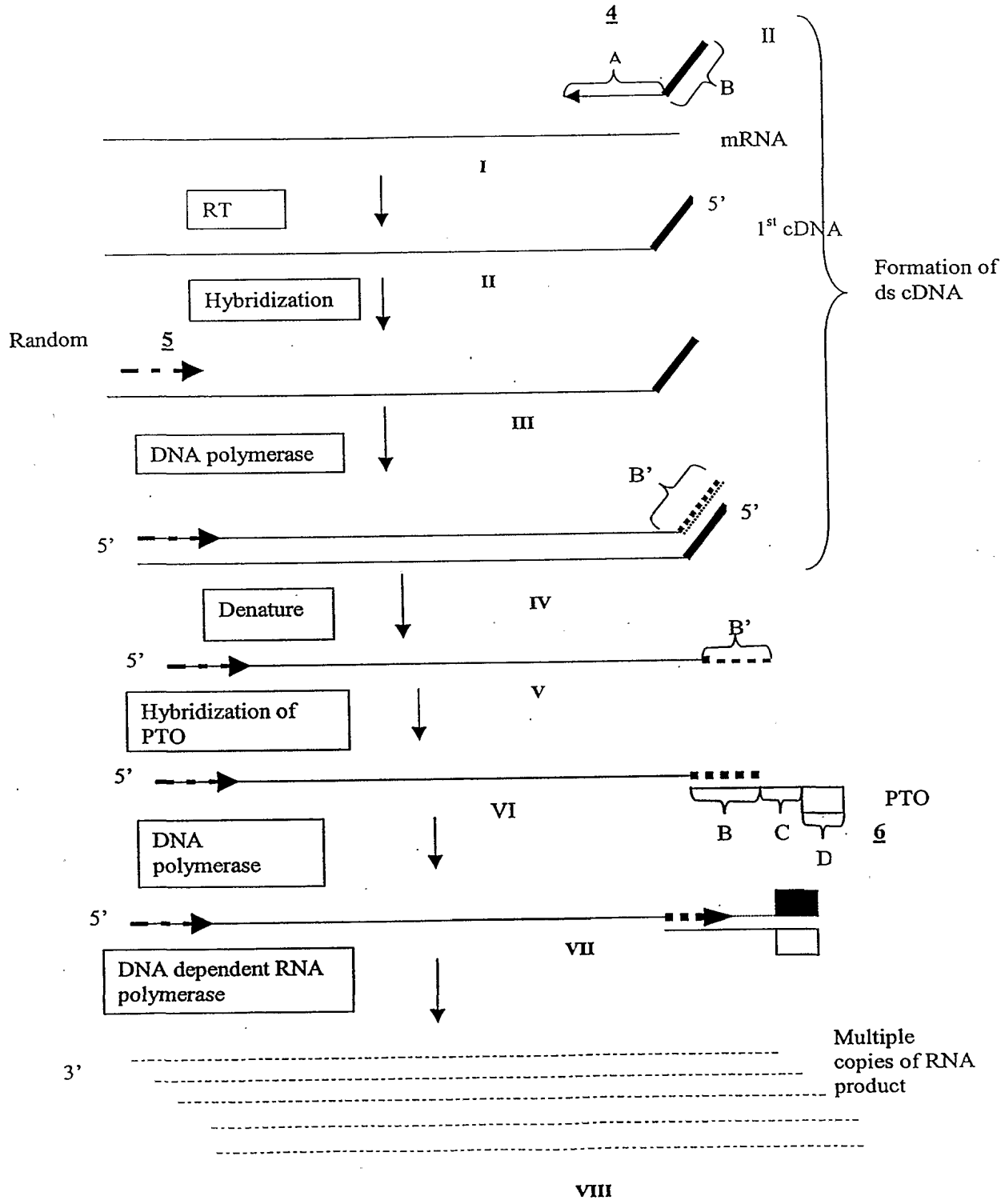


FIGURE 2A

Exponential isothermal amplification

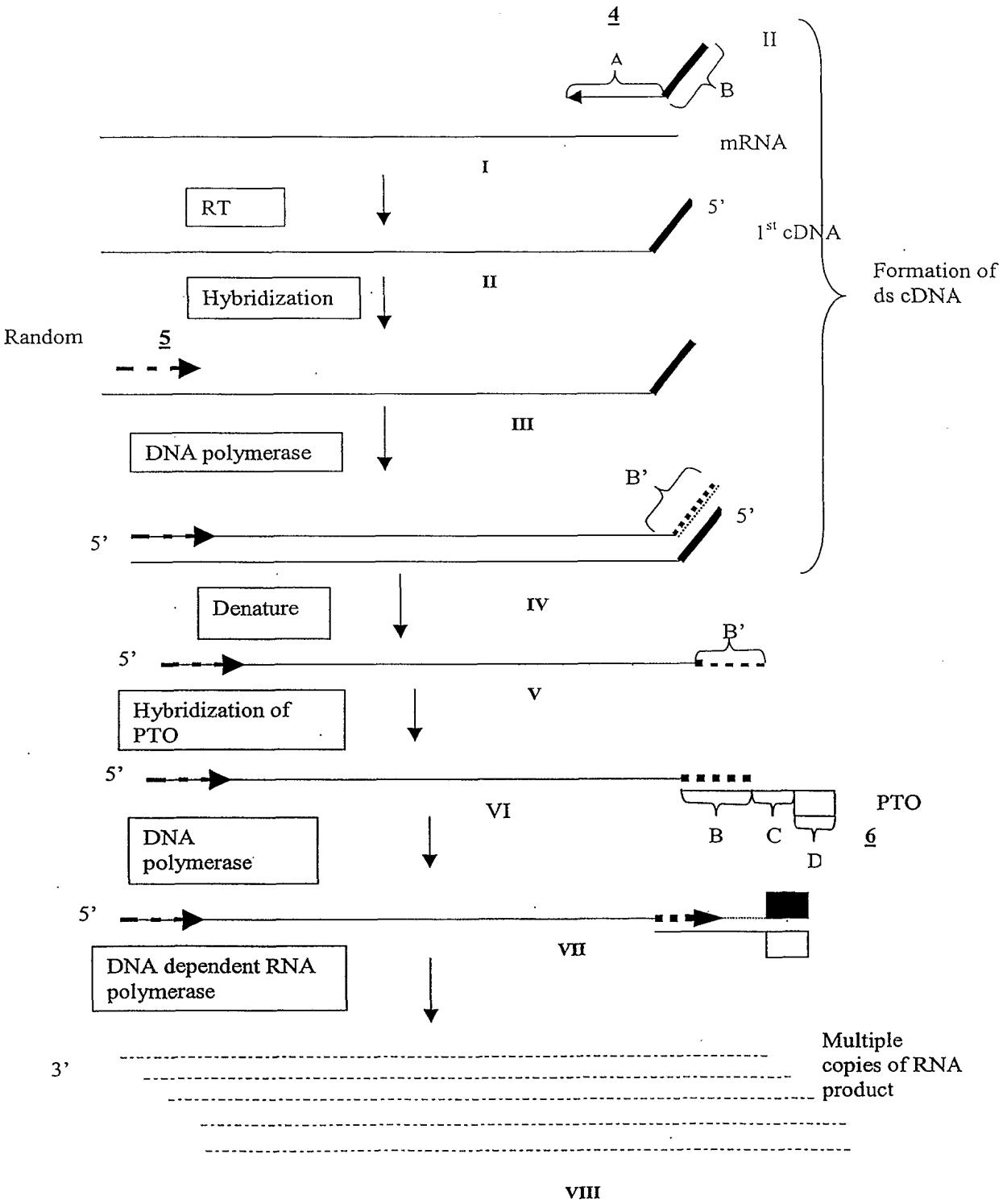


FIGURE 2B

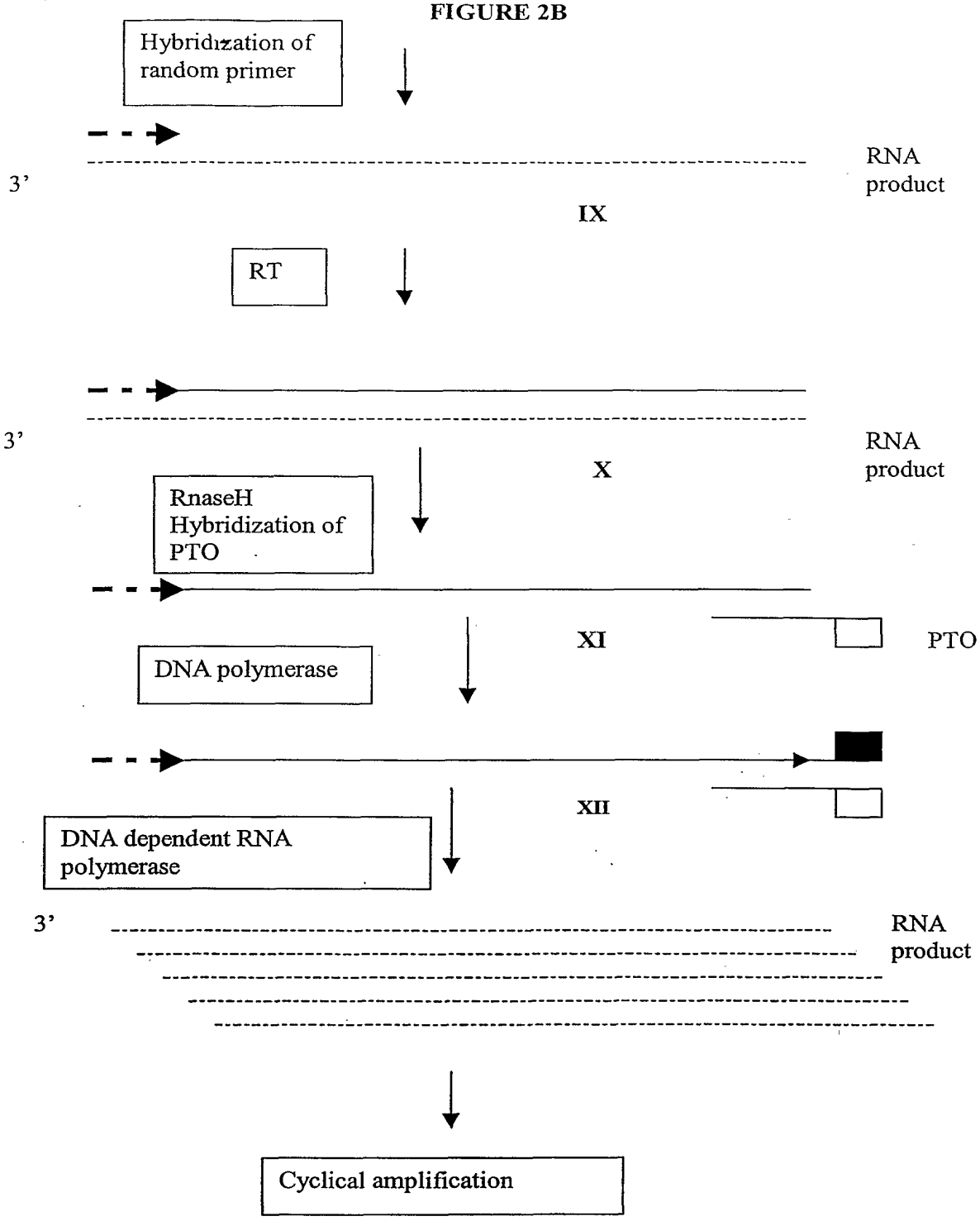

Hybridization of random primer
3'
RNA product
IX
RT
3'
RNA product
X
RnaseH Hybridization of PTO
XI
PTO
DNA polymerase
XII
DNA dependent RNA polymerase
3'
RNA product
Cyclical amplification

FIGURE 3A

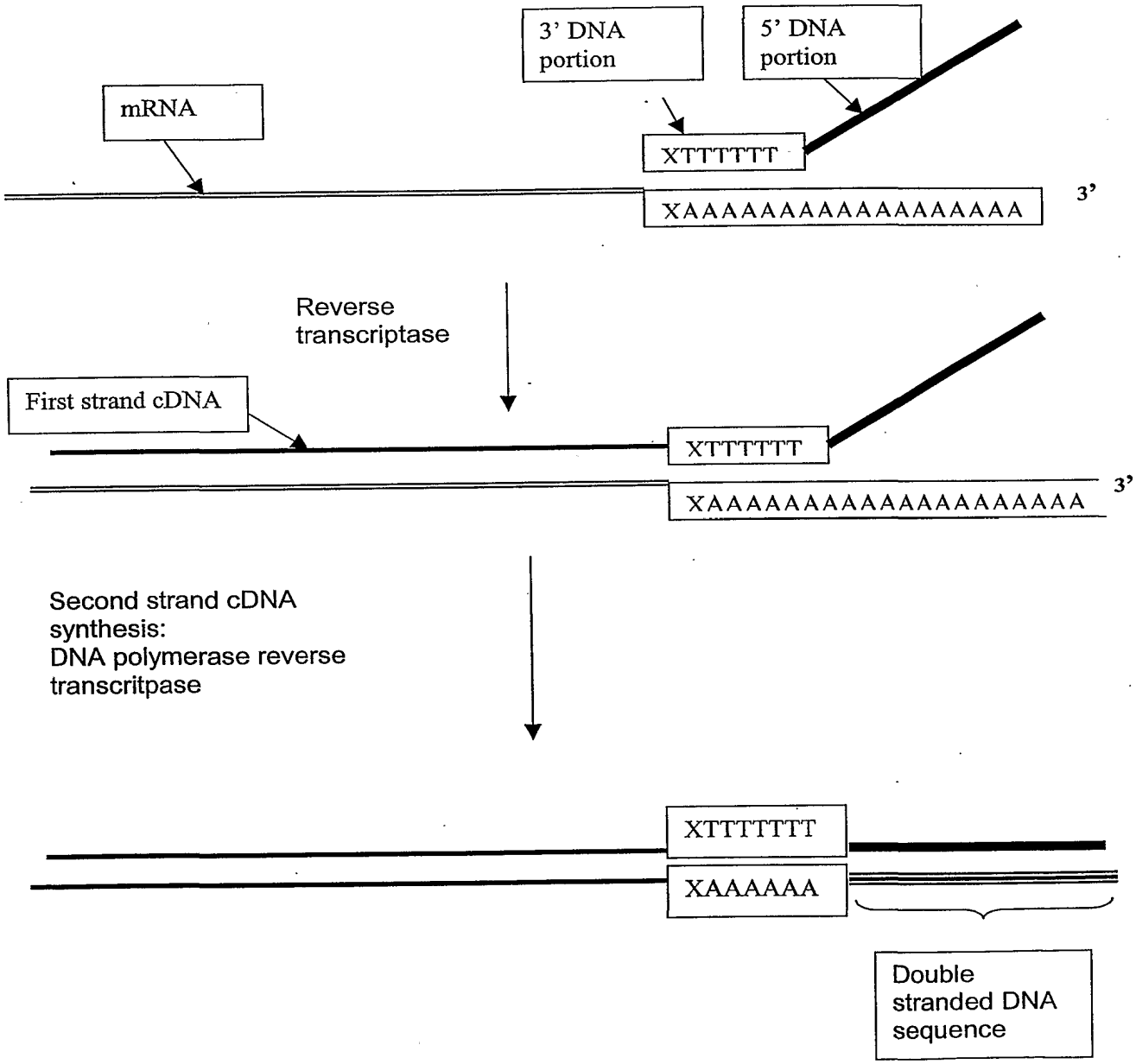
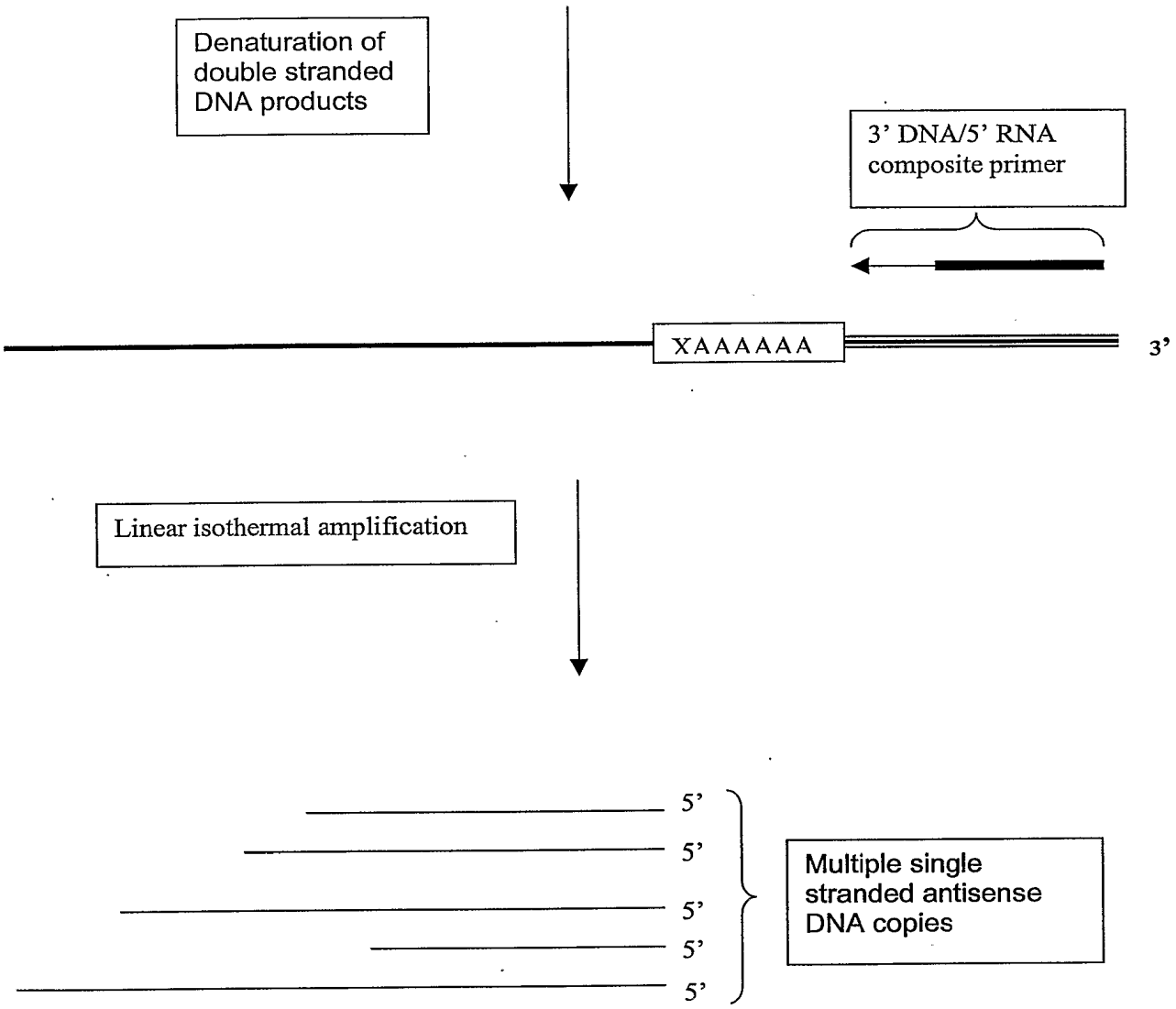

3' DNA portion
5' DNA portion
mRNA
XTTTTTT
XAAAAAAAAAAAAAAAAAA
3'
Reverse transcriptase
First strand cDNA
XTTTTTT
XAAAAAAAAAAAAAAAAAAAA
3'
Second strand cDNA synthesis:
DNA polymerase reverse transcritpase
XTTTTTTT
XAAAAAA
Double stranded DNA sequence

FIGURE 3B


Denaturation of double stranded DNA products
3' DNA/5' RNA composite primer
XAAAAAA
3'
Linear isothermal amplification
5'
5'
5'
5'
5'
Multiple single stranded antisense DNA copies

Espacenet

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ISOTHERMAL DNA AMPLIFICATION

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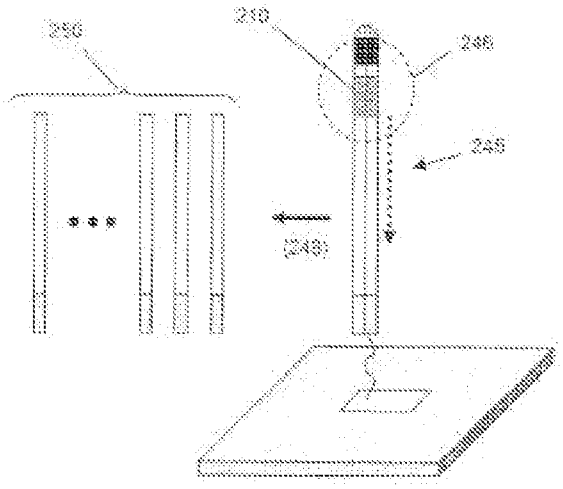
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Abstract of WO2006081222 (A2)

The present invention provides for amplification of one or more polynucleotides by multi-staged linear amplifications using one or more RNA polymerases. At each stage RNA transcripts are accumulated at a linear rate, so that multiple stages provide for faster than linear transcript accumulation. In one aspect, the invention provides for polynucleotide amplification by ligating hairpin adaptors to an end of polynucleotides wherein the hairpin adaptors each contain a promoter sequence oriented so that transcription proceeds in the direction of the loop of the hairpin adaptor. Upon transcription through such loop region and to the complementary strand a replicate is made of the promoter sequence as well as the polynucleotide, thereby permitting exponential amplification



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upon reverse transcription, second strand synthesis, and repetition of the above cycle.; Preferably such amplification is carried out under isothermal reaction conditions.

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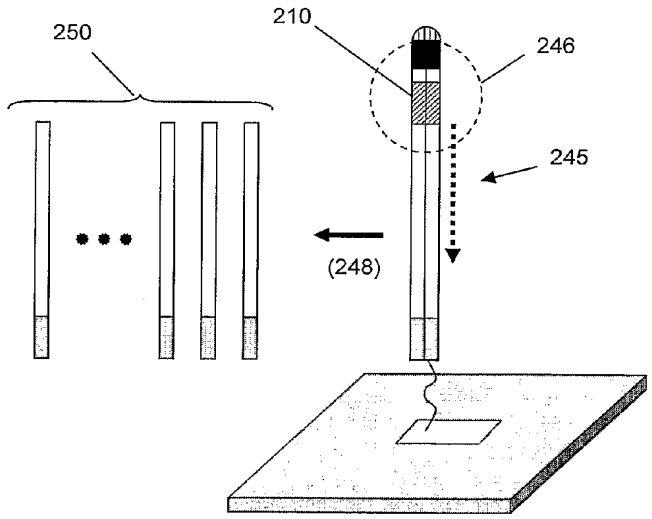
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(54) Title: ISOTHERMAL DNA AMPLIFICATION



(57) **Abstract:** The present invention provides for amplification of one or more polynucleotides by multi-staged linear amplifications using one or more RNA polymerases. At each stage RNA transcripts are accumulated at a linear rate, so that multiple stages provide for faster than linear transcript accumulation. In one aspect, the invention provides for polynucleotide amplification by ligating hairpin adaptors to an end of polynucleotides wherein the hairpin adaptors each contain a promoter sequence oriented so that transcription proceeds in the direction of the loop of the hairpin adaptor. Upon transcription through such loop region and to the complementary strand a replicate is made of the promoter sequence as well as the polynucleotide, thereby permitting exponential amplification upon reverse transcription, second strand synthesis, and repetition of the above cycle. Preferably such amplification is carried out under isothermal reaction conditions.

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ISOTHERMAL DNA AMPLIFICATION

Field of the Invention

The present invention relates to methods for amplifying polynucleotides, and more particularly, for amplifying on a solid phase support a single strand of a double stranded target DNA.

BACKGROUND

Much of the progress in genetics and biotechnology has depended on the availability of techniques for amplifying nucleic acids to produce amounts amenable for analysis, for example, by sequencing, hybridization, fluorescent labeling, or the like. A wide variety of amplification techniques have been developed for this purpose, including polymerase chain reaction (PCR), ligase chain reaction (LCR), nucleic acid sequence-based amplification (NASBA), rolling circle, and the like.

PCR has been the most widely used method to amplify DNA from small quantities of DNA molecules. Although it is efficient and generates large quantities of DNA from small aliquots of DNA template, PCR always introduces biases when it is applied to amplify complex DNA libraries. A host of factors affect the relative amplification rates of individual sequences with a library, including the preference of primer annealing sites, the length and GC content differences of the template DNAs, non-specific priming at interior sites of sequences, temporal differences in extension times, and the like. Frequently, because of such factors, the polymerization, or primer extension, step is terminated prematurely by the denaturation step of the PCR; hence, smaller fragments and low GC content fragments are preferentially amplified.

It would be very useful for many applications where nucleic amplification plays a key role, if an amplification technique were available that minimized or eliminated the above short-comings of PCR.

SUMMARY OF THE INVENTION

The present invention provides for amplification of one or more polynucleotides by multi-staged linear amplifications using one or more RNA polymerases. At each stage RNA transcripts are accumulated at a linear rate, so that multiple stages provide for faster than linear transcript accumulation. RNA transcripts at each stage are converted back into DNA to permit further production of secondary, tertiary, or higher order RNA transcripts, depending on the embodiment. In one aspect, the invention provides two stages of RNA transcript production, preferably under isothermal reaction conditions.

In one aspect, the invention is implemented by the method comprising the following steps: (i) providing a double stranded DNA having a hairpin at one end, the polynucleotide at the other end, and disposed therebetween a promoter sequence oriented so that synthesis by an RNA polymerase recognizing the promoter sequence proceeds in the direction of the hairpin; (ii) transcribing the double

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stranded DNA with an RNA polymerase that recognizes the promoter sequence to form an RNA transcript comprising copies of the promoter sequence and the polynucleotide; (iii) generating a complementary DNA from the RNA transcript; (iv) displacing a 5' end of the RNA transcript from the complementary DNA so that the hairpin is reconstituted; and (v) extending the hairpin to generate the double stranded DNA containing a reconstituted promoter sequence, the RNA polymerase recognizing the reconstituted promoter sequence and synthesizing RNA transcripts. In a preferred embodiment, the step of generating includes forming a heteroduplex of said complementary DNA and said RNA transcript and wherein said step of displacing includes treating the heteroduplex with a helicase.

In another aspect, the invention provides a method of amplifying a polynucleotide comprising the following steps: (i) providing a single stranded DNA containing a promoter sequence and a polynucleotide, the single stranded DNA having a 5' end, a 3' end, and complementary sequences at the 3' end capable of forming a hairpin having a loop region and a duplex region, the promoter sequence being disposed between the polynucleotide and the 3' end and the single stranded the single stranded DNA being attached to a solid phase support by its 5' end; (ii) extending the duplex to form a double stranded DNA that includes the polynucleotide and complement thereof and the promoter sequence and complement thereof, thereby forming an operational promoter; and (iii) generating RNA copies of the polynucleotide with an RNA polymerase that recognizes the operational promoter.

In another aspect, the above step of providing further includes providing a double strand DNA that contains in series an up-stream promoter, a first stem region, said loop region and complement thereof, a second stem region, said polynucleotide, and a primer binding site, wherein RNA copies of a strand of the double stranded DNA are generated by treating under synthesis conditions the double stranded DNA with an RNA polymerase that recognized the up-stream promoter, and wherein the RNA copies of a strand of the double stranded DNA are captured by specific hybridization of the primer binding site to a complement thereof attached to a solid phase support.

The invention further includes kits and compositions for carrying out the method of the invention. In one aspect, such kits include a composition of one or more hairpin adaptors that each contains a loop region and a double stranded region, wherein the double stranded region contains a promoter site oriented so that synthesis takes place in the direction of the loop region. In another aspect, such kits further include at least one RNA polymerases, at least one reverse transcriptases, and at least one primer (e.g. first primer), and appropriate buffers for implementing the method of the invention. In another aspect, such kits further include at least one DNA polymerase and at least one helicase and appropriate buffers for implementing the method of the invention.

The invention provides a method and compositions for polynucleotide amplification that minimizes amplification biases that are found in PCR. A priming step is eliminated in the second stranded DNA synthesis reaction by employing self-priming with the formation of an intramolecular looped structure at the 3' terminus of the first stranded DNA; thus, the priming is accurate and faster than intermolecular priming.

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BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 lists nucleotide sequences related to an example of the invention.

Figs. 2A-2D diagrammatically illustrate steps of several embodiments of the invention.

Fig. 3 shows images of gel lanes containing fragments from different steps of one embodiment of the method of the invention.

Figs. 4A and 4B show images of gel lanes containing product fragments from amplification reactions of the invention carried out for different reaction times in one embodiment of the invention.

Fig. 5 shows images of gel lanes containing products of reactions with Klenow (exo-) DNA polymerase on the amplification reaction in one embodiment of the invention.

Fig. 6 shows an image of gel lanes containing reaction components from an analysis of the reaction showing that T7 RNA polymerase can transcribe through a loop of a hairpin adaptor.

DEFINITIONS

Terms and symbols of nucleic acid chemistry, biochemistry, genetics, and molecular biology used herein follow those of standard treatises and texts in the field, e.g. Kornberg and Baker, DNA Replication, Second Edition (W.H. Freeman, New York, 1992); Lehninger, Biochemistry, Second Edition (Worth Publishers, New York, 1975); Strachan and Read, Human Molecular Genetics, Second Edition (Wiley-Liss, New York, 1999); Eckstein, editor, Oligonucleotides and Analogs: A Practical Approach (Oxford University Press, New York, 1991); Gait, editor, Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, 1984); and the like.

"Addressable" in reference to tag complements means that the nucleotide sequence, or perhaps other physical or chemical characteristics, of an end-attached probe, such as a tag complement, can be determined from its address, i.e. a one-to-one correspondence between the sequence or other property of the end-attached probe and a spatial location on, or characteristic of, the solid phase support to which it is attached. Preferably, an address of a tag complement is a spatial location, e.g. the planar coordinates of a particular region containing copies of the end-attached probe. However, end-attached probes may be addressed in other ways too, e.g. by microparticle size, shape, color, frequency of micro-transponder, or the like, e.g. Chandler et al, PCT publication WO 97/14028.

"Allele frequency" in reference to a genetic locus means the frequency of occurrence within a population of a particular nucleotide or sequence segment, or other sequence marker, such as an insertion or deletion of one or more nucleotides, or a particular sequence motif, at or within the genetic locus. In reference to the above, a population may be a population of individual from a defined group, e.g. Caucasian women over the age of 50, or a population may be a population of cells from an individual suffering from a disease or condition, such as cancer. In some contexts, an allele frequency may also refer to the frequency of sequences not identical to, or exactly complementary to, a reference sequence, or a set of reference sequences.

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"Amplicon" means the product of a polynucleotide amplification reaction. That is, it is a population of polynucleotides, usually but not necessarily double stranded, that are replicated from one or more starting sequences. The one or more starting sequences may be one or more copies of the same sequence, or it may be a mixture of different sequences. Amplicons may be produced by a variety of amplification reactions whose products are multiple replicates of one or more target nucleic acids. Generally, amplification reactions producing amplicons are "template-driven" in that base pairing of reactants, either nucleotides or oligonucleotides, have complements in a template polynucleotide that are required for the creation of reaction products. In one aspect, template-driven reactions are primer extensions with a nucleic acid polymerase or oligonucleotide ligations with a nucleic acid ligase. Such reactions include, but are not limited to, polymerase chain reactions (PCRs), linear polymerase reactions, nucleic acid sequence-based amplification (NASBAs), rolling circle amplifications, and the like, disclosed in the following references that are incorporated herein by reference: Mullis et al, U.S. patents 4,683,195; 4,965,188; 4,683,202; 4,800,159 (PCR); Gelfand et al, U.S. patent 5,210,015 (real-time PCR with "taqman" probes); Wittwer et al, U.S. patent 6,174,670; Kacian et al, U.S. patent 5,399,491 ("NASBA"); Lizardi, U.S. patent 5,854,033; Aono et al, Japanese patent publ. JP 4-262799 (rolling circle amplification); and the like. An amplification reaction may be a "real-time" amplification if a detection chemistry is available that permits a reaction product to be measured as the amplification reaction progresses, e.g. "real-time PCR" described below, or "real-time NASBA" as described in Leone et al, Nucleic Acids Research, 26: 2150-2155 (1998), and like references. As used herein, the term "amplifying" means performing an amplification reaction. A "reaction mixture" means a solution containing all the necessary reactants for performing a reaction, which may include, but not be limited to, buffering agents to maintain pH at a selected level during a reaction, salts, co-factors, scavengers, and the like. "RNA amplicon" means an amplicon of RNA molecules; that is, starting sequences, which may be RNA or DNA, give rise to RNA replicates in an amplicon.

"Complementary or substantially complementary" refers to the hybridization or base pairing or the formation of a duplex between nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between an oligonucleotide primer and a primer binding site on a single stranded nucleic acid. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate nucleotide insertions or deletions, pair with at least about 80% of the nucleotides of the other strand, usually at least about 90% to 95%, and more preferably from about 98 to 100%. Alternatively, substantial complementarity exists when an RNA or DNA strand will hybridize under selective hybridization conditions to its complement. Typically, selective hybridization will occur when there is at least about 65% complementary over a stretch of at least 14 to 25 nucleotides, preferably at least about 75%, more preferably at least about 90% complementary. See, M. Kanehisa Nucleic Acids Res. 12:203 (1984), incorporated herein by reference.

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"Duplex" means at least two oligonucleotides and/or polynucleotides that are fully or partially complementary undergo Watson-Crick type base pairing among all or most of their nucleotides so that a stable complex is formed. The terms "annealing" and "hybridization" are used interchangeably to mean the formation of a stable duplex. "Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one another such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. The term "duplex" comprehends the pairing of nucleoside analogs, such as deoxyinosine, nucleosides with 2-aminopurine bases, PNAs, and the like, that may be employed. A "mismatch" in a duplex between two oligonucleotides or polynucleotides means that a pair of nucleotides in the duplex fails to undergo Watson-Crick bonding.

"Genetic locus," or "locus" in reference to a genome or target polynucleotide, means a contiguous subregion or segment of the genome or target polynucleotide. As used herein, genetic locus, or locus, may refer to the position of a gene or portion of a gene in a genome, or it may refer to any contiguous portion of genomic sequence whether or not it is within, or associated with, a gene. Preferably, a genetic locus refers to any portion of genomic sequence from a few tens of nucleotides, e.g. 10-30, or 10-100, in length, to a few hundred nucleotides, e.g. 100-1000 or 100-500 in length, to a few thousands of nucleotide in length, e.g. 1000-10,000 or 1000-3000 in length. In some contexts, genetic locus may refer to the location of a nucleotide within a genome.

"Kit" refers to any delivery system for delivering materials or reagents for carrying out a method of the invention. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., probes, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. Such contents may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains probes.

"Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, e.g. oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon of a terminal nucleotide of one oligonucleotide with 3' carbon of another oligonucleotide. A variety of template-driven ligation reactions are described in the following references, which are incorporated by reference: Whitely et al, U.S. patent 4,883,750; Letsinger et al, U.S. patent 5,476,930; Fung et al, U.S. patent 5,593,826; Kool, U.S. patent 5,426,180; Landegren et al, U.S. patent 5,871,921; Xu and Kool, Nucleic Acids Research, 27: 875-881 (1999); Higgins et al, Methods

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in Enzymology, 68: 50-71 (1979); Engler et al, The Enzymes, 15: 3-29 (1982); and Namsaraev, U.S. patent publication 2004/0110213.

"Microarray" refers to a solid phase support having a planar surface, which carries an array of nucleic acids, each member of the array comprising identical copies of an oligonucleotide or polynucleotide immobilized to a spatially defined region or site, which does not overlap with those of other members of the array; that is, the regions or sites are spatially discrete. Spatially defined hybridization sites may additionally be "addressable" in that its location and the identity of its immobilized oligonucleotide are known or predetermined, for example, prior to its use. Typically, the oligonucleotides or polynucleotides are single stranded and are covalently attached to the solid phase support, usually by a 5'-end or a 3'-end. The density of non-overlapping regions containing nucleic acids in a microarray is typically greater than 100 per cm^2, and more preferably, greater than 1000 per cm^2. Microarray technology is disclosed in the following references that are incorporated by reference: Schena, Editor, Microarrays: A Practical Approach (IRL Press, Oxford, 2000); Southern, Current Opin. Chem. Biol., 2: 404-410 (1998); Nature Genetics Supplement, 21: 1-60 (1999). The synthesis of high-density microarrays is disclosed in the following exemplary references that are incorporated by reference: Fodor et al, U.S. patents 5,424,186; 5,744,305; 5,445,934; 6,355,432; 6,440,667 (Affymetrix, Santa Clara, CA); Cerrina et al, U.S. patent 6,375,903 (NimbleGen, Madison, WI); and "ink-jet" synthesized microarrays, e.g. disclosed in Hughes et al, Nature Biotechnology, 19: 342-347 (2001); Caren et al U.S. patent 6,323,043 (Agilent Technologies, Palo Alto, CA); and the like. As used herein, "random microarray" refers to a microarray whose spatially discrete regions of oligonucleotides or polynucleotides are not spatially addressed. That is, the identity of the attached oligonucleoties or polynucleotides is not discernable, at least initially, from its location. In one aspect, random microarrays are planar arrays of microbeads wherein each microbead has attached a single kind of hybridization tag complement, such as from a minimally cross-hybridizing set of oligonucleotides. Arrays of microbeads may be formed in a variety of ways, e.g. Brenner et al, Nature Biotechnology, 18: 630-634 (2000); Tulley et al, U.S. patent 6,133,043; Stuelpnagel et al, U.S. patent 6,396,995; Chee et al, U.S. patent 6,544,732; and the like. Likewise, after formation, microbeads, or oligonucleotides thereof, in a random array may be identified in a variety of ways, including by optical labels, e.g. fluorescent dye ratios or quantum dots, shape, sequence analysis, or the like. Hybridization conditions typically include salt concentrations of less than about 1M, more usually less than about 500 mM and less than about 200 mM. Hybridization temperatures can be as low as 5° C., but are typically greater than 22° C., more typically greater than about 30° C., and preferably in excess of about 37° C. Hybridizations are usually performed under stringent conditions, i.e. conditions under which a probe will stably hybridize to a perfectly complementary target sequence, but will not stably hybridize to sequences that have one or more mismatches. The stringency of hybridization conditions depends on several factors, such as probe sequence, probe length, temperature, salt concentration, concentration of organic solvents, such as

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formamide, and the like. How such factors are selected is usually a matter of design choice to one of ordinary skill in the art for any particular embodiment. Usually, stringent conditions are selected to be about 5° C lower than the T_m for the specific sequence for particular ionic strength and pH. Exemplary hybridization conditions include salt concentration of at least 0.01 M to no more than 1 M Na ion concentration (or other salts) at a pH 7.0 to 8.3 and a temperature of at least 25° C. Additional exemplary hybridization conditions include the following: 5×SSPE (750 mM NaCl, 50 mM sodium phosphate, 5 mM EDTA, pH 7.4). Exemplary hybridization procedures for applying labeled target sequence to a GenFlex™ microarray (Affymetrix, Santa Clara, CA) is as follows: denatured labeled target sequence at 95– 100°C for 10 minutes and snap cool on ice for 2-5 minutes. The microarray is pre-hybridized with 6X SSPE-T (0.9 M NaCl 60 mM NaH_2,PO_4, 6 mM EDTA (pH 7.4), 0.005% Triton X-100) + 0.5 mg/ml of BSA for a few minutes, then hybridized with 120 µL hybridization solution (as described below) at 42°C for 2 hours on a rotisserie, at 40 RPM. Hybridization Solution consists of 3M TMACL (Tetramethylammonium. Chloride), 50 mM MES ((2-[N-Morpholino]ethanesulfonic acid) Sodium Salt) (pH 6.7), 0.01% of Triton X-100, 0.1 mg/ml of Herring Sperm DNA, optionally 50 pM of fluorescein-labeled control oligonucleotide, 0.5 mg/ml of BSA (Sigma) and labeled target sequences in a total reaction volume of about 120 µL. The microarray is rinsed twice with 1X SSPE-T for about 10 seconds at room temperature, then washed with 1X SSPE-T for 15-20 minutes at 40°C on a rotisserie, at 40 RPM. The microarray is then washed 10 times with 6X SSPE-T at 22°C on a fluidic station (e.g. model FS400, Affymetrix, Santa Clara, CA). Further processing steps may be required depending on the nature of the label(s) employed, e.g. direct or indirect. Microarrays containing labeled target sequences may be scanned on a confocal scanner (such as available commercially from Affymetrix) with a resolution of 60-70 pixels per feature and filters and other settings as appropriate for the labels employed. GeneChip Software (Affymetrix) may be used to convert the image files into digitized files for further data analysis.

"Mismatch" means a base pair between any two of the bases A, T (or U for RNA), G, and C other than the Watson-Crick base pairs G-C and A-T. The eight possible mismatches are A-A, T-T, G-G, C-C, T-G, C-A, T-C, and A-G.

"Mutation" and "polymorphism" are usually used somewhat interchangeably to mean a DNA molecule, such as a gene that differs in nucleotide sequence from a reference DNA sequence or wild type sequence, or normal tissue sequence, by one or more bases, insertions, and/or deletions. In some contexts, the usage of Cotton (Mutation Detection, Oxford University Press, Oxford, 1997) is followed in that a mutation is understood to be any base change whether pathological to an organism or not, whereas a polymorphism is usually understood to be a base change with no direct pathological consequences.

"Nucleoside" as used herein includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having

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modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543-584 (1990), or the like, with the proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like. Polynucleotides comprising analogs with enhanced hybridization or nuclease resistance properties are described in Uhlman and Peyman (cited above); Crooke et al, Exp. Opin. Ther. Patents, 6: 855-870 (1996); Mesmaeker et al, Current Opinion in Structual Biology, 5: 343-355 (1995); and the like. Exemplary types of polynucleotides that are capable of enhancing duplex stability include oligonucleotide N3'→P5' phosphoramidates (referred to herein as "amidates"), peptide nucleic acids (referred to herein as "PNAs"), oligo-2'-O-alkylribonucleotides, polynucleotides containing C-5 propynylpyrimidines, locked nucleic acids (LNAs), and like compounds. Such oligonucleotides are either available commercially or may be synthesized using methods described in the literature.

"Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one other such that every nucleotide in each strand undergoes Watson-Crick basepairing with a nucleotide in the other strand. The term also comprehends the pairing of nucleoside analogs, such as deoxyinosine, nucleosides with 2-aminopurine bases, and the like, that may be employed. In reference to a triplex, the term means that the triplex consists of a perfectly matched duplex and a third strand in which every nucleotide undergoes Hoogsteen or reverse Hoogsteen association with a basepair of the perfectly matched duplex. Conversely, a "mismatch" in a duplex between a tag and an oligonucleotide means that a pair or triplet of nucleotides in the duplex or triplex fails to undergo Watson-Crick and/or Hoogsteen and/or reverse Hoogsteen bonding.

"Polymerase chain reaction," or "PCR," means a reaction for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. In other words, PCR is a reaction for making multiple copies or replicates of a target nucleic acid flanked by primer binding sites, such reaction comprising one or more repetitions of the following steps: (i) denaturing the target nucleic acid, (ii) annealing primers to the primer binding sites, and (iii) extending the primers by a nucleic acid polymerase in the presence of nucleoside triphosphates. Usually, the reaction is cycled through different temperatures optimized for each step in a thermal cycler instrument. Particular temperatures, durations at each step, and rates of change between steps depend on many factors well-known to those of ordinary skill in the art, e.g. exemplified by the references: McPherson et al, editors, PCR: A Practical Approach and PCR2: A Practical Approach (IRL Press, Oxford, 1991 and 1995, respectively). For example, in a conventional PCR using Taq DNA polymerase, a double stranded target nucleic acid may be denatured at a temperature >90°C, primers annealed at a temperature in the range 50-75°C, and primers extended at a temperature in the range 72-78°C. The term "PCR" encompasses derivative forms of the reaction, including but not limited to, RT-PCR, real-time PCR, nested PCR, quantitative PCR, multiplexed PCR, and the like. Reaction volumes range from a few hundred nanoliters, e.g. 200 nL, to a few hundred μL, e.g. 200

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μL. "Reverse transcription PCR," or "RT-PCR," means a PCR that is preceded by a reverse transcription reaction that converts a target RNA to a complementary single stranded DNA, which is then amplified, e.g. Tecott et al, U.S. patent 5,168,038, which patent is incorporated herein by reference. "Real-time PCR" means a PCR for which the amount of reaction product, i.e. amplicon, is monitored as the reaction proceeds. There are many forms of real-time PCR that differ mainly in the detection chemistries used for monitoring the reaction product, e.g. Gelfand et al, U.S. patent 5,210,015 ("taqman"); Wittwer et al, U.S. patents 6,174,670 and 6,569,627 (intercalating dyes); Tyagi et al, U.S. patent 5,925,517 (molecular beacons); which patents are incorporated herein by reference. Detection chemistries for real-time PCR are reviewed in Mackay et al, Nucleic Acids Research, 30: 1292-1305 (2002), which is also incorporated herein by reference. "Nested PCR" means a two-stage PCR wherein the amplicon of a first PCR becomes the sample for a second PCR using a new set of primers, at least one of which binds to an interior location of the first amplicon. As used herein, "initial primers" in reference to a nested amplification reaction mean the primers used to generate a first amplicon, and "secondary primers" mean the one or more primers used to generate a second, or nested, amplicon. "Multiplexed PCR" means a PCR wherein multiple target sequences (or a single target sequence and one or more reference sequences) are simultaneously carried out in the same reaction mixture, e.g. Bernard et al, Anal. Biochem., 273: 221-228 (1999)(two-color real-time PCR). Usually, distinct sets of primers are employed for each sequence being amplified. "Quantitative PCR" means a PCR designed to measure the abundance of one or more specific target sequences in a sample or specimen. Quantitative PCR includes both absolute quantitation and relative quantitation of such target sequences. Quantitative measurements are made using one or more reference sequences that may be assayed separately or together with a target sequence. The reference sequence may be endogenous or exogenous to a sample or specimen, and in the latter case, may comprise one or more competitor templates. Typical endogenous reference sequences include segments of transcripts of the following genes: β-actin, GAPDH, β_2-microglobulin, ribosomal RNA, and the like. Techniques for quantitative PCR are well-known to those of ordinary skill in the art, as exemplified in the following references that are incorporated by reference: Freeman et al, Biotechniques, 26: 112-126 (1999); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9447 (1989); Zimmerman et al, Biotechniques, 21: 268-279 (1996); Diviacco et al, Gene, 122: 3013-3020 (1992); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9446 (1989); and the like.

"Polynucleotide" or "oligonucleotide" are used interchangeably and each mean a linear polymer of nucleotide monomers. Monomers making up polynucleotides and oligonucleotides are capable of specifically binding to a natural polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Such monomers and their internucleosidic linkages may be naturally occurring or may be analogs thereof, e.g. naturally occurring or non-naturally occurring analogs. Non-naturally occurring analogs may include PNAs, phosphorothioate internucleosidic linkages, bases containing linking groups permitting the attachment of labels, such

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as fluorophores, or haptens, and the like. Whenever the use of an oligonucleotide or polynucleotide requires enzymatic processing, such as extension by a polymerase, ligation by a ligase, or the like, one of ordinary skill would understand that oligonucleotides or polynucleotides in those instances would not contain certain analogs of internucleosidic linkages, sugar moities, or bases at any or some positions. Polynucleotides typically range in size from a few monomeric units, e.g. 5-40, when they are usually referred to as "oligonucleotides," to several thousand monomeric units. Whenever a polynucleotide or oligonucleotide is represented by a sequence of letters (upper or lower case), such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, "I" denotes deoxyinosine, "U" denotes uridine, unless otherwise indicated or obvious from context. Unless otherwise noted the terminology and atom numbering conventions will follow those disclosed in Strachan and Read, Human Molecular Genetics 2 (Wiley-Liss, New York, 1999). Usually polynucleotides comprise the four natural nucleosides (e.g. deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine for DNA or their ribose counterparts for RNA) linked by phosphodiester linkages; however, they may also comprise non-natural nucleotide analogs, e.g. including modified bases, sugars, or internucleosidic linkages. It is clear to those skilled in the art that where an enzyme has specific oligonucleotide or polynucleotide substrate requirements for activity, e.g. single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for the oligonucleotide or polynucleotide substrates is well within the knowledge of one of ordinary skill, especially with guidance from treatises, such as Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references.

"Primer" means an oligonucleotide, either natural or synthetic that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. Extension of a primer is usually carried out with a nucleic acid polymerase, such as a DNA or RNA polymerase. The sequence of nucleotides added in the extension process is determined by the sequence of the template polynucleotide. Usually primers are extended by a DNA polymerase. Primers usually have a length in the range of from 14 to 40 nucleotides, or in the range of from 18 to 36 nucleotides. Primers are employed in a variety of nucleic amplification reactions, for example, linear amplification reactions using a single primer, or polymerase chain reactions, employing two or more primers. Guidance for selecting the lengths and sequences of primers for particular applications is well known to those of ordinary skill in the art, as evidenced by the following references that are incorporated by reference: Dieffenbach, editor, PCR Primer: A Laboratory Manual, 2nd Edition (Cold Spring Harbor Press, New York, 2003).

"Readout" means a parameter, or parameters, which are measured and/or detected that can be converted to a number or value. In some contexts, readout may refer to an actual numerical representation of such collected or recorded data. For example, a readout of fluorescent intensity

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signals from a microarray is the address and fluorescence intensity of a signal being generated at each hybridization site of the microarray; thus, such a readout may be registered or stored in various ways, for example, as an image of the microarray, as a table of numbers, or the like. A readout is "digital" when the number or value is obtained by a counting process, e.g. determining a value by counting on a microarray the number of hybridization from which signals are being generated (as distinguished from those sites not generating signals).

"Sample" means a quantity of material from a biological, environmental, medical, or patient source in which detection or measurement of target nucleic acids is sought. On the one hand it is meant to include a specimen or culture (e.g., microbiological cultures). On the other hand, it is meant to include both biological and environmental samples. A sample may include a specimen of synthetic origin. Biological samples may be animal, including human, fluid, solid (e.g., stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may include materials taken from a patient including, but not limited to cultures, blood, saliva, cerebral spinal fluid, pleural fluid, milk, lymph, sputum, semen, needle aspirates, and the like. Biological samples may be obtained from all of the various families of domestic animals, as well as feral or wild animals, including, but not limited to, such animals as ungulates, bear, fish, rodents, etc. Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

"Solid support", "support", and "solid phase support" are used interchangeably and refer to a material or group of materials having a rigid or semi-rigid surface or surfaces. In many embodiments, at least one surface of the solid support will be substantially flat, although in some embodiments it may be desirable to physically separate synthesis regions for different compounds with, for example, wells, raised regions, pins, etched trenches, or the like. According to other embodiments, the solid support(s) will take the form of beads, resins, gels, microspheres, or other geometric configurations. Microarrays usually comprise at least one planar solid phase support, such as a glass microscope slide.

"Specific" or "specificity" in reference to the binding of one molecule to another molecule, such as a labeled target sequence for a probe, means the recognition, contact, and formation of a stable complex between the two molecules, together with substantially less recognition, contact, or complex formation of that molecule with other molecules. In one aspect, "specific" in reference to the binding of a first molecule to a second molecule means that to the extent the first molecule recognizes and forms a complex with another molecules in a reaction or sample, it forms the largest number of the complexes with the second molecule. Preferably, this largest number is at least fifty percent. Generally, molecules involved in a specific binding event have areas on their surfaces or in cavities giving rise to specific recognition between the molecules binding to each other. Examples of specific binding include antibody-antigen interactions, enzyme-substrate interactions, formation of duplexes or

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triplexes among polynucleotides and/or oligonucleotides, receptor-ligand interactions, and the like. As used herein, "contact" in reference to specificity or specific binding means two molecules are close enough that weak noncovalent chemical interactions, such as Van der Waal forces, hydrogen bonding, base-stacking interactions, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules.

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the Tm of nucleic acids are well known in the art. As indicated by standard references, a simple estimate of the Tm value may be calculated by the equation. Tm = 81.5 + 0.41 (% G + C), when a nucleic acid is in aqueous solution at 1 M NaCl (see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985). Other references (e.g., Allawi, H.T. & SantaLucia, J., Jr., Biochemistry 36, 10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of Tm.

"Vector" or "cloning vector" refers to an extrachromosomal genetic element which can be used to replicate a DNA fragment in a host organism. A wide variety of cloning vectors are commercially available for use with the invention, e.g. New England Biolabs (Beverely, Mass.); Stratagene Cloning Systems (La Jolla, Calif.); Clontech Laboratories (Palo Alto, Calif.); and the like. Usually, cloning vectors used with the invention are bacterial plasmids.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a method for amplifying, or replicating, one or more polynucleotides in a reaction. In one aspect, the method converts a strand of a DNA duplex into an RNA amplicon in a multi-stage reaction that exponentially increases the number of replicate molecules with time. In accordance with the invention, replication in each stage of the reaction is accomplished by providing a promoter site in an operational configuration with the polynucleotide to be amplified, or a replicate of it. Thus, at each stage, each replicate is linearly amplified for a combined amplification rate across all the reaction stages that is greater than linear. In another aspect, the methods of the invention are carried out under isothermal reaction conditions. In still another aspect, methods of the invention can be implemented on a solid phase support to create amplicons containing a single kind of molecule, which amplicons are restricted to discrete locations on a surface of such solid phase support, e.g., as illustrated below, by hybridizing to complementary oligonucleotides attached to the surface at such discrete locations. Below, exemplary embodiments are described that implement the principles of the invention. In a first embodiment, a two-stage reaction is described in which two different promoters, each recognized by a different RNA polymerase, are used in a different stage of an overall reaction. In a second embodiment, a two-stage reaction is described in which a single promoter is used in two different reaction stages for amplification. An important feature is the use of a first stand cDNA that

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can form a hairpin structure at its 3' end to provide a self-priming template for second strand synthesis.

In one embodiment, such amplification is carried out in an isothermal reaction using components as illustrated in Figs. 2A and 2B. Double stranded DNA (200) is provided having the following elements: up-stream promoter (202), first stem region (204), loop region (206), second stem region (208), promoter (210), polynucleotide (212), and primer binding site (214). Double stranded DNA (200) is treated (218) under synthesis conditions with RNA polymerase (216) that recognized up-stream promoter (202) so that RNA copies (220) are produced of the sequence downstream of up-stream promoter. Synthesis conditions depend on the particular RNA polymerase used, but usually are the manufacturer's recommended conditions, which minimally include the four ribonucleoside triphosphates and appropriate buffers. Exemplary RNA polymerases include T7 RNA polymerase, T3 RNA polymerase, SP6 RNA polymerase, and the like. RNA copies (220) are annealed by way of primer binding site (214) to primer (224) attached by its 5' end to solid phase support (226). Primer (224) and primer binding site (214) have sufficient complementarity to form a stable duplex that may be extend with a reverse transcriptase (RT), such as avian myeloblastosis virus RT, Moloney murine leukemia virus, or the like. Primer (224) may be attached to solid phase support (226) via a biotin, or equivalent capture moiety, attached to its 5' nucleotide. Alternatively, primer (226) may be directly synthesized on the surface of solid phase support (226) using 5'-phosphoramidite chemistry (e.g. Glen Research), or the like. After a stable duplex forms between primer (224) and primer binding site (214), primer (224) is treated (228) under synthesis conditions with reverse transcriptase (230) to form DNA strand (233) from RNA template (235). After such reaction is completed, RNA template (235) may be removed (236) chemically or enzymatically by conventional means, e.g. heating in sodium hydroxide solution or by RNase H treatment, after which first stem region (204) may anneal to second stem region (208) to form an extendable duplex. Such extendable duplex is treated under synthesizing conditions with DNA polymerase (242) to form (244) double stranded DNA (245), after which double stranded DNA (245) is treated under synthesis conditions with RNA polymerase that recognizes promoter (210). Such reaction (248) produces RNA transcripts (250). Optionally, RNA transcripts (250) may be converted into DNA annealing a primer to primer binding site (214) and extending the primer with a reverse transcriptase along RNA transcripts (250).

In another embodiment, the method of the invention is implemented using a single promoter sequence by reversing its orientation relative to that of the previous embodiment and ligating a hairpin adaptor containing it to the polynucleotide to be amplified. Part of the invention is the discovery that RNA polymerases recognizing a promoter oriented to promote synthesis in the direction of a hairpin loop are able to synthesize an RNA transcript across the loop and along the complementary strand of the promoter sequence itself. As illustrated in Fig. 2C, hairpin adaptor (252) comprising loop (255), promoter sequence (253), and, preferably overhang (254) (to facilitate ligation) is ligated to a complementary overhang on an end of polynucleotide (256). Polynucleotide (256) preferably

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includes a primer binding site at its end opposite to that of the hairpin adaptor (252), e.g. similar to (214) of Fig. 2A. In embodiments where multiple polynucleotides are amplified, preferably the polynucleotides all have a common primer binding site. After ligation, augmented sequence (257) is formed, which is treated (258) with RNA polymerase (259) that recognizes promoter sequence (253). Upon binding to promoter sequence (253), RNA polymerase (259) initiates synthesis of an RNA transcript along a template that includes loop (255). Such RNA transcripts accumulate at a linear rate with time to form an initial RNA amplicon (260). To the sequences in this amplicon are added first primers (263), which in the presence of reverse transcriptase (262) and the four nucleoside triphosphates are extended so that each forms a first cDNA strand as part of RNA-DNA heteroduplex (264). In one version of this embodiment, strand displacing primers (266) are added in order to primer synthesis of a second DNA strand by DNA polymerase (265). Such strand displacing primers may be made from, or contain, peptide nucleic acids, or like nucleotide analogs that are capable of forming duplexes with enhanced stability. Alternatively, the RNA strand can be digested with RNase H. After double stranded DNA (280) is formed, a second stage of RNA replication is initiated by RNA polymerase (259) at the reconstituted promoter sequence (253), which results in the generation of a secondary RNA amplicon (268). Another version of this embodiment is shown in Fig. 2D. The two versions proceed similarly up to the formation of heteroduplex (264). In the second version, ends (275) and (277) of heteroduplex (264) are unwound by helicase (270) so that stem region (278) is regenerated, thereby providing an extendable duplex. Useful helicases are described in Vincent et al, EMBO Reports, 5:795-800 (2004), and in U.S. patent publication 2004/0058378, which are incorporated by reference. Preferably, a sen1p helicase is employed in the invention, such as disclosed in Kim et al, Biochemistry, 38: 14697-14710 (1999), and Ursic et al, Nucleic Acids Research, 32: 2441-2452 (2004), both of which are incorporated by reference. The extendable duplex is extended by DNA polymerase (265) to form double stranded DNA (282) containing promoter sequence (253), which is identical to augmented sequence (257). Thus, when RNA polymerase (259) binds to promoter sequence (253) synthesis again proceeds towards loop (284) to generate secondary RNA amplicon (268).

Example 1

Solid Phase Capture and Amplification

In this example, T7 RNA polymerase copies a template DNA to form RNA transcripts, which are captured by a unique primer and then utilized as templates to synthesize the first stranded complementary DNAs. This first strand DNA flips back at the 3'terminus to provide entry point for self-polymerization, because the 3'terminus has complementary sequence to form loop and stem structure. A T3 RNA polymerase promoter in the sequence is then reconstituted after self-polymerization. After the first in vitro transcription-reverse transcriptase (IVT-RT) mediated amplification reaction, the looped double stranded DNA serves as template in the second IVT-RT reaction to transcribe more RNA. In this embodiment, RNA is removed from the heteroduplex prior

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to the self-priming reaction by denaturation (i.e. in alkaline conditions or rising the temperature). Once the 3' end of cDNA is exposed as single stranded, looped double stranded DNA is synthesized on the solid surface as well as in the liquid phase by the DNA dependent DNA polymerase activity of reverse transcriptase. This looped double stranded DNA on the solid surface serves as template for IVT by T3 RNA polymerase. The transcript from T3 promoter is captured by the same capture primer. The amplification fold on the solid surface is the function of the amount of input double stranded DNA, the amount of immobilized primer to capture transcript, and the free capture primer in the second IVT-RT reaction. The amplification fold, which depends on the amount of input DNA, was estimated to be from 10 to 20000 when the second IVT-RT reaction was carried out at 37°C for 1 to 3 hours incubation under conditions where no non-specific amplification is observed.

Structure of template for amplification. The consensus sequences of promoters T7 (Dunn and Studier (1983) J. Mol. Biol., 166: 477-535) and T3 (Beck et al (1989) J. Mol. Biol., 210: 687-701) are employed in the template to be amplified (fig.1A). GC pairs were placed at –18 and –19 position of T7 promoter sequence to minimize separation or "breathing" of the T7 promoter sequence at the end. The T7 leader sequence was changed from GGGAGA to GGGATT to introduce a loop and stem structure at the 3' end of first strand cDNA. Two restriction enzymes NcoI and NheI sites were located just after the T3 promoter leader sequence to permit library construction and amplification of the sequences. A tag sequence can be located after the NheI site to identify each insert fragment. Because of the difficulty in synthesizing long oligonucleotides, the length of tag sequence was reduced from five words to one word. This shorter version of template DNA was used. An 11-mer sequence was placed at the very end of the right hand side of the template for primer annealing site to permit cDNA synthesis using a reverse transcriptase. This same sequence was used for immobilize cDNA on the solid surface.

The first stranded DNA synthesis and IVT conditions. The first stranded DNA synthesis and in vitro transcription reactions were combined in one reaction. Reverse transcriptase requires potassium ion, which inhibits RNA polymerase activity when it is used in excess. On the other hand, RNA polymerase requires spermidine, which inhibits reverse transcriptase activity when it is used in excess. 2 mM spermidine and 50 mM KCl were used to facilitate both activities in one reaction. The IVT-RT reaction was performed in 20 µl reaction volume containing 50 mM KCl, 40 mM Tris-HCl (pH 8), 16 mM MgCl2, 10 mM DTT, 2 mM spermidine and 0.1 mg/ml BSA supplied with 1 mM dNTPs, 2 mM rNTPs, 0.1 pmol of template double stranded DNA and 1 µM 5' labeled fluorescent primer with Bodipy630/650 which anneals to the 3' terminus of transcript, along with RNase inhibitor, 0.5 U/µl T7 RNA polymerase and 5 U/µl Superscript II reverse transcriptase. The same fluorescent primer was used in all of experiments of this example. The IVT-RT reaction mixture was incubated at 37°C for four hours. After incubation, aliquot was taken and mixed with blue dextran denaturing dye, denatured at 100°C for 5 minutes and analyzed on 8 % PAGE denaturing urea gel,

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which was run at 40 W constant for 35 min. Storm860 was used to visualize gel image and analyzed it with ImageQuant NT software.

Liquid phase amplification. The RNA-DNA heteroduplex obtained above was denatured by rising the temperature to 100 degree for 5 min to expose the single stranded 3' end of the cDNA strand for self polymerization. This enabled synthesis of the template for the IVT reaction and reverse transcription reaction simultaneously. The second IVT-RT reaction was performed under the same conditions except that instead of T7 RNA polymerase, T3 RNA polymerase was used. The second IVT-RT reaction contains 50 mM KCl, 40 mM Tris-HCl (pH 8), 16 mM MgCl2, 10 mM DTT, 2 mM spermidine and 0.1 mg/ml BSA supplied with 1 mM dNTPs, 2 mM rNTPs, an aliquot of looped single stranded DNA and 1 μM 5' labeled fluorescent primer which anneals to the 3' terminus of transcript along with RNase inhibitor, 0.5 U/μl T3 RNA polymerase and 5 U/μl Superscript II reverse transcriptase in a 20 μL reaction volume. This IVT-RT reaction mixture was incubated at 37°C for one hour.

Solid phase amplification. A Streptavidin coated polystyrene plate (Reacti-Bind Streptavidin High Binding Capacity Coated Plates, PIERCE) was used as a solid support to immobilize biotinylated primer to capture transcripts from T7 promoter in the first IVT-RT reaction. 200 pmol of primer in100 μl 1 x BW buffer (5 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1.0 M NaCl) was used for coating the plate well with biotinylated primer at room temperature for one hour incubation. After binding primer on the wells, wells were washed two times with 1x BW buffer, once with water, once with T7' buffer (40 mM Tris-HCl (pH 8), 6 mM MgCl2) prior to the first IVT-RT reaction. The IVT-RT reaction was performed in 40 μL reaction volume containing 50 mM KCl, 40 mM Tris-HCl (pH 8), 16 mM MgCl2, 10 mM DTT, 2 mM spermidine, 1 mg/ml heparin and 0.1 mg/ml BSA supplied with 1 mM dNTPs, 2 mM rNTPs, various amount of template double stranded DNA along with RNase inhibitor, 0.5 U/μl T7 RNA polymerase and 5 U/μl Superscript II reverse transcriptase. This IVT-RT reaction mixture was incubated at 37°C for four hours. After four hours incubation, wells were washed with 1 x T7' buffer two times, once with water, then RNA striping solution (0.5 M NaOH, 1.5 M NaCl) was added to the wells and incubated at room temperature for 10 to 15 min. Wells were washed with once with water, neutralized with 1 M Tris-HCl (pH 7.0) two times, washed with water once, washed with 10 mM Tris-HCl (pH 8.0) once and then 1 x T7' buffer two times prior to the second IVT-RT reactions. The second IVT-RT reaction was performed in 40 μl reaction volume containing 50 mM KCl, 40 mM Tris-HCl (pH 8), 16 mM MgCl2, 10 mM DTT, 2 mM spermidine and 0.1 mg/ml BSA supplied with 1 mM dNTPs, 2 mM rNTPs and 1 μM 5' labeled fluorescent primer along with RNase inhibitor, 0.5 U/μL T3 RNA polymerase and 5 U/μl Superscript II reverse transcriptase. The reactions were incubated at 37°C and 1 μL aliquots were drown from the reactions at appropriate time and mixed with 3 μLof blue dextran denaturing dye, denature at 100 deg for 5 min prior to apply samples on 8 % Polyacrylamide gel (fig.4).

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Estimation of amplification fold on the solid surface. The amount of final amplified DNA in the polystyrene well was estimated by the consumption of the fluorescent labeled oligonucleotide. The total capture oligonucleotide used in the reaction is 40 pmol in 40 μl second IVT-RT reaction volume. The final amount of amplified DNA is totally dependent on the amount of this capture oligo. Various amounts of input DNA (0.001 pmol to 1 pmol) were used in the first IVT-RT reaction. The incubation time for the first IVT-RT reaction was fixed at four hours. After removing RNA from first stranded DNA attached to the polystyrene surface, second IVT-RT cocktails were added to each well and 1 μL reactions were carried out. Samples were denatured with blue dextran denaturing dye and analyzed on 8 % PAGE denaturing gel. The signal intensity was quantified by ImageQuantNT. As 1 μl sample at T = 0 shows the initial amount of capture oligo, which is 1 pmol, the amounts of amplified cDNA (Dout) is estimated by Dout = IcDNA / (IcDNA + Icap) x 1 pmol, where IcDNA is the signal intensity of amplified cDNA and Icap is the signal intensity of unincorporated capture oligonucleotide. The amplification fold is defined by Dout / Din. Dout and Din are the total amount of estimated output DNA and input DNA, respectively.

Displacement of the looped RNA structure at its 5'terminus by reverse transcriptase. Full length cDNA synthesis was carried out, as the amplification scheme requires formation of a hairpin at the 3' end of the first stranded cDNA for providing self priming to regenerate a double stranded DNA. Fig.3 (lane 4 and lane 5) shows the position of the reverse transcribed first stranded cDNA from IVT-RT reaction compared with the size of the chemically synthesized full-length cDNA (fig3 lane1). The size of this chemically synthesized oligonucleotide is 72 nucleotides and that of the first stranded cDNA is expected to be 72 nucleotides (fig.1). The band size on lane 4 and 5 indicate that the full-length cDNA is synthesized. Although the 5' terminus of transcript has looped structure, this structure is unfolded and stretched to serve as a template to make full-length cDNA.

Removing RNA from heteroduplex to give looped DNA with 3' end for the entry point of DNA polymerase and reconstitution of an active T3 promoter. Although the looped structure of transcript at its 5' terminus is stretched, or linearized, by reverse transcriptase (fig.3 lane 4 and lane 5), the heteroduplex is sufficiently stable to prevent the formation of looped DNA at its 3' end. The size of the first stranded cDNA is not elongated more than the size of its full-length cDNA in the IVT-RT reaction. rGTP was substituted with rITP to reduce the hydrogen bonding in the heteroduplex; however, no looped double stranded DNA was observed. Since the synthesized single stranded DNA, which contains looped structure at its 3' terminus, is able to utilize the looped 3' terminus as an entry point for polymerization by self priming (fig.3 lane 2), single stranded cDNA striped off from the RNA-DNA heteroduplex can be self primed. Therefore, RNA was removed from heteroduplex by RNaseH treatment followed by heat denaturation in the liquid phase amplification or alkaline denaturation in the solid phase amplification. After such treatment, the single stranded synthesized DNA was capable to provide 3' priming point by self-annealing at its 3' terminus (fig.3 lane 6). Once the RNA was removed from the heteroduplex, single stranded DNA self primed and was copied by

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Klenow(exo-) DNA polymerase and/or SSII. This looped double stranded DNA reconstitute an active T3 promoter from which a transcript of the expected size was produced (fig.3 lane 8 to lane 10). Although the reaction run on lane 7 did not contain T7RNA polymerase and SSII in the first IVT-RT reaction (fig.3 lane 3), a weak signal was observed on lane 7. This weak signal was derived from the initial input double stranded DNA carried over to the second IVT-RT reaction. Thus, the labeled DNA on lane 8 to lane 10 migrated at the same position at which the weak band is observed on lane 7. This indicates that the size of transcripts were the same, as they were transcribed from the same T3 promoter.

Degree of amplification on the solid surface. The prolonged incubation time in the second IVT-RT reaction leads to the consumption of the free capture primers in the capture primer uncoated well (fig.4A and 4B). When there is no capture primer in the well, looped double stranded DNA cannot be synthesized (fig.1 B). Background cDNA synthesis in the second IVT-RT reaction is due to the non-specific binding of the initial input DNA to the well in the first IVT-RT reaction. There are two possible scenarios can be considered for this non-specific amplification. The first one is non-specific bond DNA on the well is carried over to the second IVT-RT reaction and the second one is that transcript is carried over and served as the template for cDNA synthesis in the second IVT-RT reaction. However, the second case is very unlikely because of the following reasons. If the T7 transcripts is carried over after denaturation process to the second IVT-RT reaction and this transcript is utilized to synthesize cDNA, T3 promoter is still inactive because heteroduplex is too stable to give 3' self priming site. Thus it is very unlikely that the T7 transcript carried over is a template in the second IVT-RT reaction that caused non-specific amplification. This was confirmed by employing an initial template with an inactive T3 promoter sequence by substituting dATP with diaminopurine, which suppresses the back ground cDNA synthesis. If the back ground amplification is suppressed, more capture primer and precursors will be consumed only by the transcript derived from double stranded looped DNA, which will make the amplification fold be increased. The amplification fold was estimated for each input DNA at the maximal incubation time which does not cause any back ground amplification. The less the input DNA is used, the higher the amplification fold was observed (table 1). The amount of final amplified cDNA was restricted to the total amount of free capture primer in the second IVT-RT reaction, which is 40 pmol in the experiments. As no more than 40 pmol of cDNA synthesis can be expected, the maximal amplification is 40 when 1 pmol of input DNA was used. What is more, the higher amount of input DNA increased the back ground amplification before the IVT reaction achieved saturation state. Thus, the less input DNA gives higher amplification fold. Although, the minimal amount of input DNA used was 25 amol / μl, it can be decreed further, in which case a higher amplification fold more than 20,000 can be expected.

The effect of Klenow (exo-) DNA polymerase in the amplification reaction. Figure 5 shows the effect of using Klenow (exo-) DNA polymerase in the liquid phase amplification reaction. The first IVT-RT reaction was performed at 37°C for 4 hours followed by second IVT-RT reaction for one

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hour. In the absence of T7RNA polymerase and RT in the first IVT-RT reaction, 1 pmol template was required to see a weak signal in the second IVT-RT reaction in the absence of Klenow (exo-) DNA polymerase (fig. 5 lane 6). In the presence of T7RNA polymerase and RT in the first IVT-RT reaction, minimal amount of the template to be detected after amplification is 0.01 pmol (fig.5 lane 14). The addition of Klenow (exo-) DNA polymerase to the second IVT-RT reaction increased the amount of amplified product (fig. 5 lane 7 to lane 10 and lane 17 to lane 20). This might be because the polymerization efficiency of Klenow (exo-) DNA polymerase is higher than that of RT, which enables the production of more looped double stranded DNA in the second IVT-RT reaction (fig. 5 lane 13 to lane 20). However, this enhancement was also observed under the conditions when no first stranded DNA was synthesized (lane 7 to 10). The first IVT-RT reaction did not show any significant effect on the amplification fold in the second IVT-RT reaction in the presence of Klenow (exo-) DNA polymerase. Even 0.001 pmol of template in the absence of T7RNA polymerase and RT showed the amplified product (Fig. 5 lane 7). The size of these products on lane 7 to lane 10 indicates that they are obtained from RNA transcribed from the T3 promoter on the initial DNA as a template. Thus, the effect of enhancement of Klenow (exo-) DNA polymerase is mainly on the first stranded cDNA synthesis from the T3 transcript as a template. The enhancement of final product by Klenow (exo-) DNA polymerase probably due to the rapid and recurrent entry of the primer to the annealing site on the T3 transcript by the strand displacement activity of Klenow (exo-) DNA polymerase rather than efficient polymerization activity.

Although DNA dependent DNA polymerase activity of reverse transcriptase was used for preparing template in the second IVT-RT reaction, an increase in the amplification fold might be accomplished by employing the strand displacement activity of Klenow (exo-) DNA polymerase in the IVT-RT reaction for the template DNA lacking active T3 promoter.

Double stranded DNA can be amplified isothermically by the combination of IVT and RT reactions. Transcript from the T7 promoter is primed and served as a template to synthesize full length cDNA. Although, 5' end of transcript has a stem and loop structure which potentially can truncate cDNA synthesis, the reverse transcriptase displace the 5'looped RNA structure and copied it to the end of the template strand. Preferably RNA is removed from the heteroduplex to permit formation of the 3' looped DNA structure, otherwise the heteroduplex is too stable to permit the formation of a 3' stem and loop structure for self priming. Once RNA is removed, the remaining sequence self primed efficiently and RT elongate the priming site to the end to reconstitute the T3 promoter. This looped double stranded DNA served as a template for the second IVT reaction. The input double stranded DNA is amplified as anti-sense single stranded DNA.

Figure 1 *Structure of the template sequence to be amplified.* (A. Input DNA) T7 and T3 promoter sequences are shown in bold. Stem and loop structure are between these two promoter sequences. Stem region is underlined and loop region is represented in small letters. After T3 promoter leader sequence, *Noc*I and *Nhe*I sites are located, which are indicated in italicized small letters. The sequence for annealing capture primer is shown in bold lowercase on the right. (B. pT7

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Transcript) The transcript from T7 promoter is shown. 5'end stem and loop structures are underlined and in small letters, respectively. The size of full-length cDNA (C) is 72 mer, and that of looped double stranded DNA (D) is 131-mer.

Figure 2 *Amplification steps*. Din and Dout represent the input double stranded DNA and the output single stranded DNA, which is the anti-sense strand of the input DNA. R7 is the transcript from T7 promoter, which anneals to the immobilized capture primer (Cimm) on the solid surface. 5' end of Cimm is attached to the solid surface. R7Dc is heteroduplex form between T7 transcript and the full-length cDNA. Dc is the single stranded full-length cDNA attached to the solid surface after R7 is removed. Dloop is the looped double stranded DNA, which serves as template for IVT by T3 RNA polymerase to transcribe R3. R3 is annealed with free capture primer (Cfree) in the solution and form R3Cfree. Cfree 3' end is extended by RT to form heteroduplex R3Dout.

Figure 3 *First and second IVT-RT reaction*. The transcript in the first IVT-RT reaction was annealed to the fluorescent labeled capture primer. Reverse transcription reaction and second IVT reactions were performed in the liquid phase. First lane shows the position of the chemically synthesized 72 mer DNA, and the second lane shows the position of the looped double stranded DNA, whose size is 131-mer, synthesized from the same DNA run on the first lane by Klenow (exo-) DNA polymerase. Lane 3 to lane 6 shows the first IVT-RT reactions with different enzyme combinations. The reaction run on lane 3 did not contain any enzymes. The reaction on lane 4 contains T7 RNA polymerase and SSII, the reaction on lane 5 and lane 6 contains T7 RNA polymerase, SSII and RNaseH. All the reactions were heat denatured. The reaction run on lane 6 was followed by polymerization with Klenow (exo-) DNA polymerase after heat denaturation. Aliquots were taken from the reactions run on lane 4 to lane 6 and added to the second IVT-RT reaction, each aliquots from second IVT-RT reactions were run on from lane 7 to lane 10, respectively.

Figure 4 *Time course of amplification reaction*. The Streptavidin coated polystyrene plate were charged with (lanes in even number) or without (lanes in odd number) capture primer. After first IVT-RT reactions in the well, wells were washed and second IVT-RT reaction mixtures were added to each well. 1 μl of aliquots of reactions were drown from each well at indicated time (in min) shown on the bottom of each lane. The total amount of input DNA used in the first IVT-RT reaction was 0.01 pmol in (A) and 0.1 pmol in (B), respectively.

Figure 5 *The effect of Klenow (exo-) DNA polymerase in the amplification reaction*. Lane 1 to 10 shows the reaction in the absence of T7 RNA polymerase and RT in the first IVT-RT reaction. The reactions on lane 1 and lane 2 did not contain T3 RNA polymerase nor RT. Lane 3 to lane 10 shows the reaction in the presence of T3 RNA polymerase and RT in the second IVT-RT reaction. Klenow (exo-) DNA polymerase was added to the reactions shown on lane 7 to lane 10 along with T3 RNA polymerase and RT. Lane 11 to lane 20 shows the reaction in the presence of T7 RNA polymerase and RT in the first IVT-RT reaction. The reaction on lane 11 and lane 12 did not contain T3 RNA polymerase nor RT. Lane 13 to lane 20 shows the reaction in the presence of T3 RNA polymerase and RT in the second IVT-RT reaction. Klenow (exo-) DNA polymerase was added to

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the reactions shown on lane 17 to lane 20 along with T3 RNA polymerase and RT. The band observed on lane 12 is the full-length first stranded DNA. The total amount of template used in the 20 μl reaction is as follows: lane 1 and 11, 1 fmol, lane 2 and12, 1 pmol, lane 3, 7, 13 and 17, 0.001 pmol, lane 4, 8, 14 and 18, 0.01 pmol, lane 5, 9, 15 and 19, 0.1 pmol, lane 6, 10, 16 and 20, 1 pmol, respectively.

Table 1 *Amplification fold on the solid surface.* The amplification fold was estimated at the maximum incubation time Tmax when back ground amplification (odd lanes on fig. 4 A and B) was not observed. The total amount of input template DNA (Din) and the estimated total amount of output DNA (DNAout) are shown on the table.

Example 2

Amplification with a Single RNA Polymerase

In this example, a hairpin adaptor containing a single promoter site was ligated to an end of the polynucleotide to be amplified (thereby forming an augmented sequence). The promoter was oriented in the hairpin adaptor so that transcription takes place in the direction of the loop, thereby creating transcripts containing a copy of the loop, a single strand of the promoter, and the polynucleotide. The sequence of the hairpin adaptor (R6) is as follows (SEQ ID NO: 5):

5′-pATCCCTTGCT[pT7]GGGAGACCCTATCTTAGGGTCTCCC[pT7′]AGCAAGG

where p is a phosphate group, [pT7] is the T7 promoter sequence, and [pT7'] is the complement of the T7 promoter sequence, the double underlined nucleotides are the loop region, and the first G of the single underlined nucleotides is the first copied nucleotide of a T7-generated transcript. After ligation, the augmented sequence was transcribed with T7 RNA polymerase under conventional reaction conditions to generate RNA transcripts. The reaction components were analyzed by gel electrophoresis, the image of which is shown in Fig. 6. Lane 1 contains the polynucleotide to be amplified before ligation with the hairpin adaptor. Lane 2 contains the product of the ligation reaction where the hairpin adaptor was ligated to the polynucleotide to form the augmented sequence. Lane 3 shows the same product after DNase I treatment. Lane 4 contains the IVT reaction product formed by treating the product of lane 2 with T7 RNA polymerase. Lane 5 contains the same product of lane 4 after DNase I treatment. Lane 4 show that full length transcription through the loop was achieved.

The above teachings are intended to illustrate the invention and do not by their details limit the scope of the claims of the invention. While preferred illustrative embodiments of the present invention are described, it will be apparent to one skilled in the art that various changes and modifications may be made therein without departing from the invention, and it is intended in the appended claims to cover all such changes and modifications that fall within the true spirit and scope of the invention.

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We claim:

1. A method of amplifying a polynucleotide comprising the steps of:

providing a single stranded DNA containing a promoter sequence and a polynucleotide, the single stranded DNA having a 5' end, a 3' end, and complementary sequences at the 3' end capable of forming a hairpin having a loop and a duplex, the promoter sequence being disposed between the polynucleotide and the 3' end and the single stranded DNA being attached to a solid phase support by its 5' end;

extending the duplex to form a double stranded DNA that includes the polynucleotide and complement thereof and the promoter sequence and complement thereof, thereby forming an operational promoter; and

generating RNA copies of the polynucleotide with an RNA polymerase that recognizes the operational promoter.

2. The method of claim 1 wherein said step of providing further includes providing a double strand DNA that contains in series an up-stream promoter, a first stem region, said loop region and complement thereof, a second stem region, said polynucleotide, and a primer binding site, wherein RNA copies of a strand of the double stranded DNA are generated by treating under synthesis conditions the double stranded DNA with an RNA polymerase that recognized the up-stream promoter, and wherein the RNA copies of a strand of the double stranded DNA are captured by specific hybridization of the primer binding site to a complement thereof attached to a solid phase support.

3. The method of claim 2 wherein said step of providing further includes treating under synthesis conditions said complement of said primer binding site with a reverse transcriptase to form said single stranded DNA.

4. The method of claim 3 said step of generating further including a step of converting said RNA copies into single stranded DNA by annealing a primer to said primer binding site and treating under synthesis conditions such primer with a reverse transcriptase.

5. The method according to claims 4 further including a step of converting to single stranded DNA said RNA copies of said polynucleotide by annealing a primer to said primer binding site and treating under synthesis conditions such primer with a reverse transcriptase.

6. A method of amplifying a strand of a polynucleotide, the method comprising the steps of:

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ligating to an end of the polynucleotide a hairpin adaptor to form an augmented sequence, the hairpin adaptor having a loop region at one end and a duplex region containing a promoter sequence oriented so that transcription proceeds toward the loop region;

combining the augmented sequence, an RNA polymerase, a reverse transcriptase, a DNA polymerase, a first primer specific for the polynucleotide, and a helicase under reaction conditions such that the RNA polymerase generates transcripts that contain the promoter sequence, the first primers anneal to the transcripts and are extended by the reverse transcriptase to produce a heteroduplex containing a first complementary DNA strand, the heteroduplex is unwound at an end such that the loop region and the duplex region are regenerated in the first complementary DNA strand and the duplex region is extended by the DNA polymerase to reconstitute an augmented sequence, the RNA polymerase recognizing the promoter sequence of the reconstituted augmented sequence and synthesizing transcripts of the polynucleotide.

7. The method of claim 6 wherein said loop region contains from three to six nucleotides.

8. The method of claim 6 wherein said polynucleotide has an end distal to said end that said hairpin adaptor is ligated and wherein said distal end said polynucleotide has a primer binding site specific for said first primers.

9. A method of amplifying a polynucleotide in a reaction, the method comprising the steps of:

providing a first double stranded DNA having a hairpin at one end, the polynucleotide at the other end, and disposed therebetween a promoter sequence oriented so that synthesis by an RNA polymerase recognizing the promoter sequence proceeds in the direction of the hairpin;

combining the double stranded DNA, an RNA polymerase, a reverse transcriptase, a DNA polymerase, a first primer specific for the polynucleotide, and a helicase under reaction conditions such that the RNA polymerase generates primary transcripts that contain the promoter sequence, the first primers anneal to the primary transcripts and are extended by the reverse transcriptase to produce a heteroduplex containing a complementary DNA strand, the heteroduplex is unwound at an end such that the hairpin is regenerated in the complementary DNA strand and is extended by the DNA polymerase to form an augmented sequence having a reconstituted promoter sequence, the RNA polymerase recognizing the reconstituted promoter sequence and synthesizing transcripts of the polynucleotide.

10. The method of claim 9 wherein said hairpin has a loop region that contains from three to six nucleotides.

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11. The method of claim 10 wherein said polynucleotide has an end distal to said end that said hairpin and wherein said distal end said polynucleotide has a primer binding site specific for said first primers.

12. The method of claim 11 wherein said step of combining includes treating said heteroduplex by a helicase to unwind said heteroduplex.

13. A method of replicating a polynucleotide, the method comprising the steps of:

providing a double stranded DNA having a hairpin at one end, the polynucleotide at the other end, and disposed therebetween a promoter sequence oriented so that synthesis by an RNA polymerase recognizing the promoter sequence proceeds in the direction of the hairpin;

transcribing the double stranded DNA with an RNA polymerase that recognizes the promoter sequence to form an RNA transcript comprising copies of the promoter sequence and the polynucleotide;

generating a complementary DNA from the RNA transcript;

displacing a 5' end of the RNA transcript from the complementary DNA so that the hairpin is reconstituted; and

extending the hairpin to generate the double stranded DNA containing a reconstituted promoter sequence, the RNA polymerase recognizing the reconstituted promoter sequence and synthesizing RNA transcripts.

14. The method of claim 13 wherein said step of generating includes forming a heteroduplex of said complementary DNA and said RNA transcript and wherein said step of displacing includes treating the heteroduplex with a helicase.

15. The method of claim 13 wherein said loop region contains from three to six nucleotides.

16. The method of claim 13 wherein said polynucleotide has an end distal to said end that said hairpin adaptor is ligated and wherein said distal end said polynucleotide has a primer binding site specific for said first primers.

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Input DNA (100)(SEQ ID NO: 1)

5′-GC**TAATACGACTCACTATA**GGGATTCTATCCC**AATTAACCCTCACTAAA**GGGAGACCATGGTCTTGCTAGCCTTGAACCG**TCGATTGGCTT**

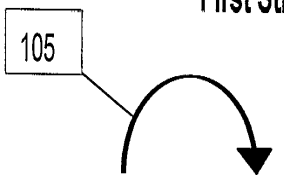
CG**ATTATGCTGAGTGATAT**CCCTAAGATAGGG**TTAATTGGGAGTGATTT**CCCTCTGGTACCAGAACGATCGGAACTTGGC**AGCTAACCGAA**

T7 promoter Stem-Loop T3 promoter NcoI NheI Tag Cap

T7 Transcript (102)(SEQ ID NO: 2)

5′-GGGAUUCUAUCCC**AAUUAACCCUCACUAAA**GGGAGACCAUGGUCUUGCUAGCCUUGAACCG**UCGAUUGGCUU**

First Strand cDNA and Self Priming 3' End (104)(SEQ ID NO: 3)



3′-CCCTAAGATAGGG**TTAATTGGGAGTGATTT**CCCTCTGGTACCAGAACGATCGGAACTTGGC**AGCTAACCGAA**

Reconstitution of Active T3 Promoter (106)(SEQ ID NO: 4)

G AATCCC**AATTAACCCTCACTAAA**GGGAGACCATGGTCTTGCTAGCCTTGAACCGTCGATTGGCTT-3′

ATAGGG**TTAATTGGGAGTGATTT**CCCTCTGGTACCAGAACGATCGGAACTTGGCAGCTAACCGAA-5′

Fig. 1

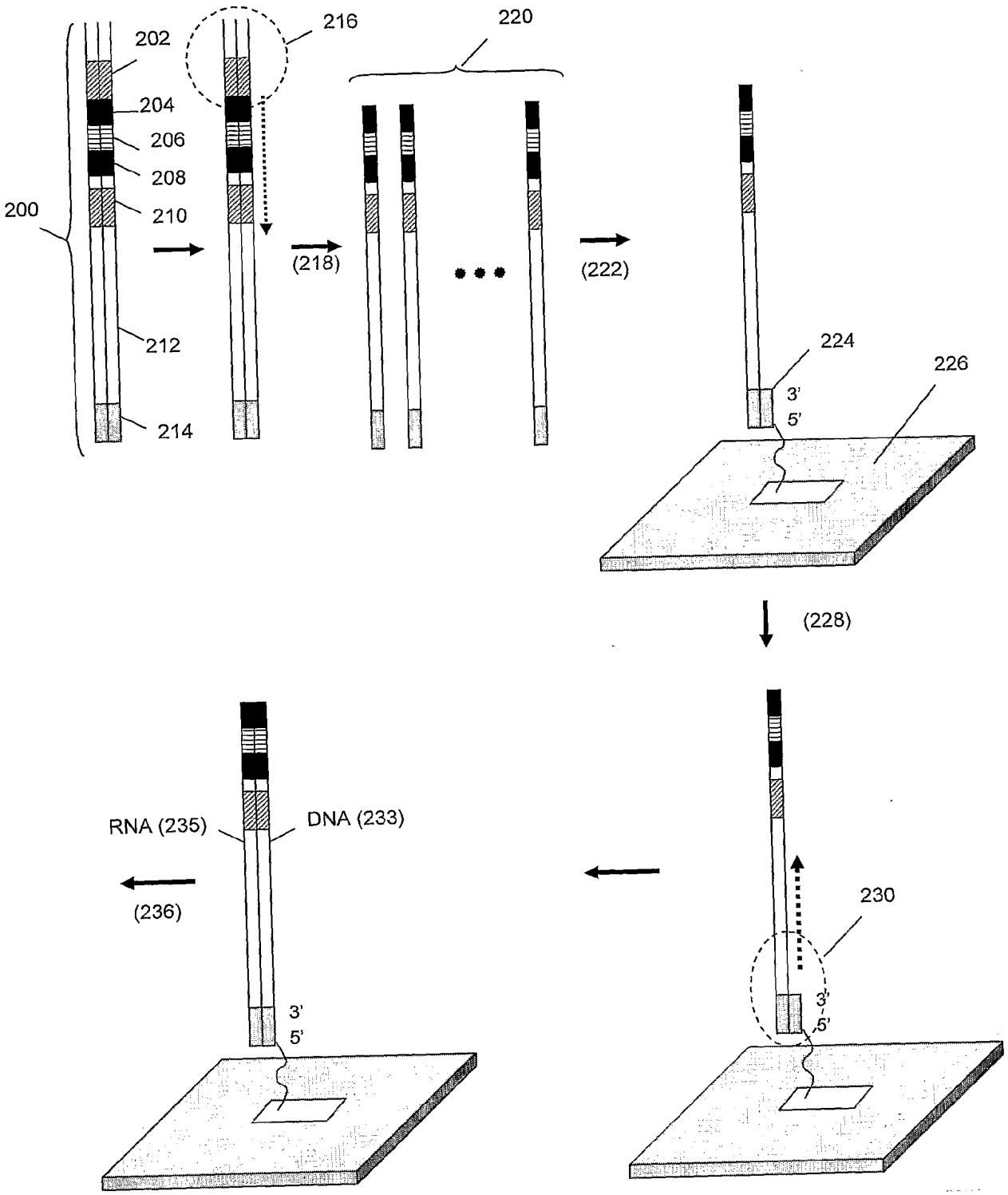

202
216
220
204
206
208
210
200
(218)
(222)
212
214
224
226
3'
5'
(228)
RNA (235)
DNA (233)
(236)
230
3'
5'

Fig. 2A

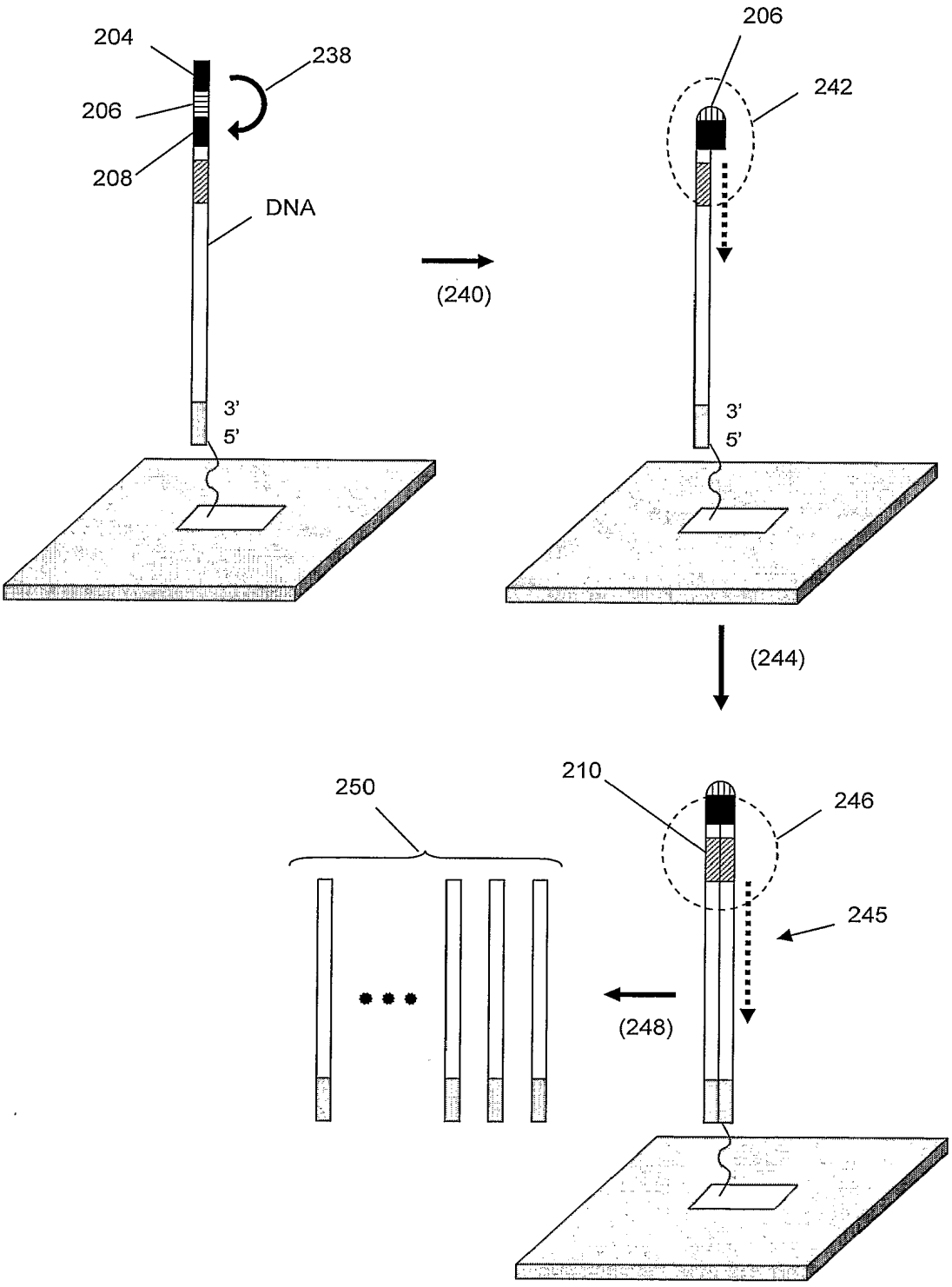


Fig. 2B

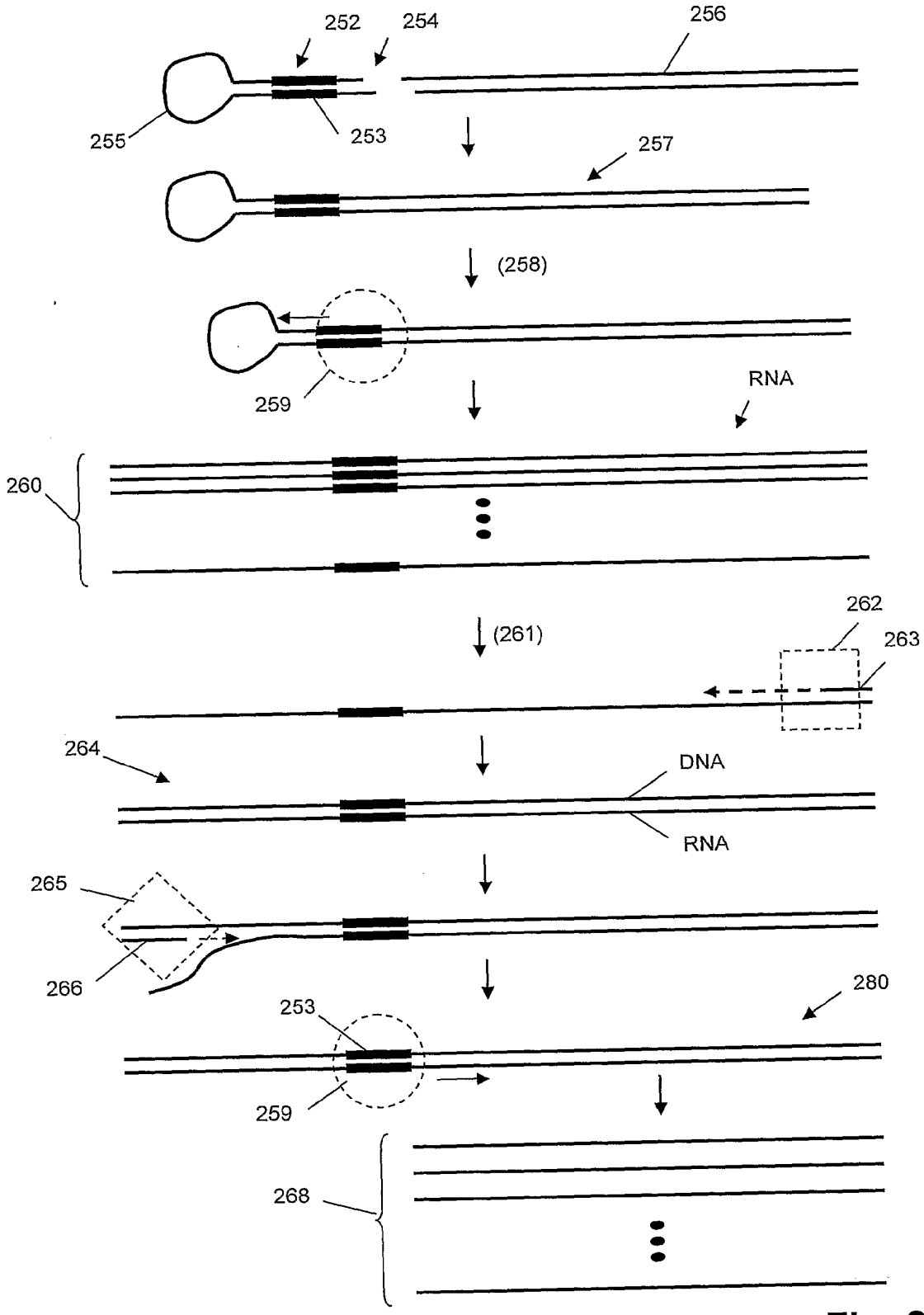

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253
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RNA
260
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DNA
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266
280
253
259
268

Fig. 2C

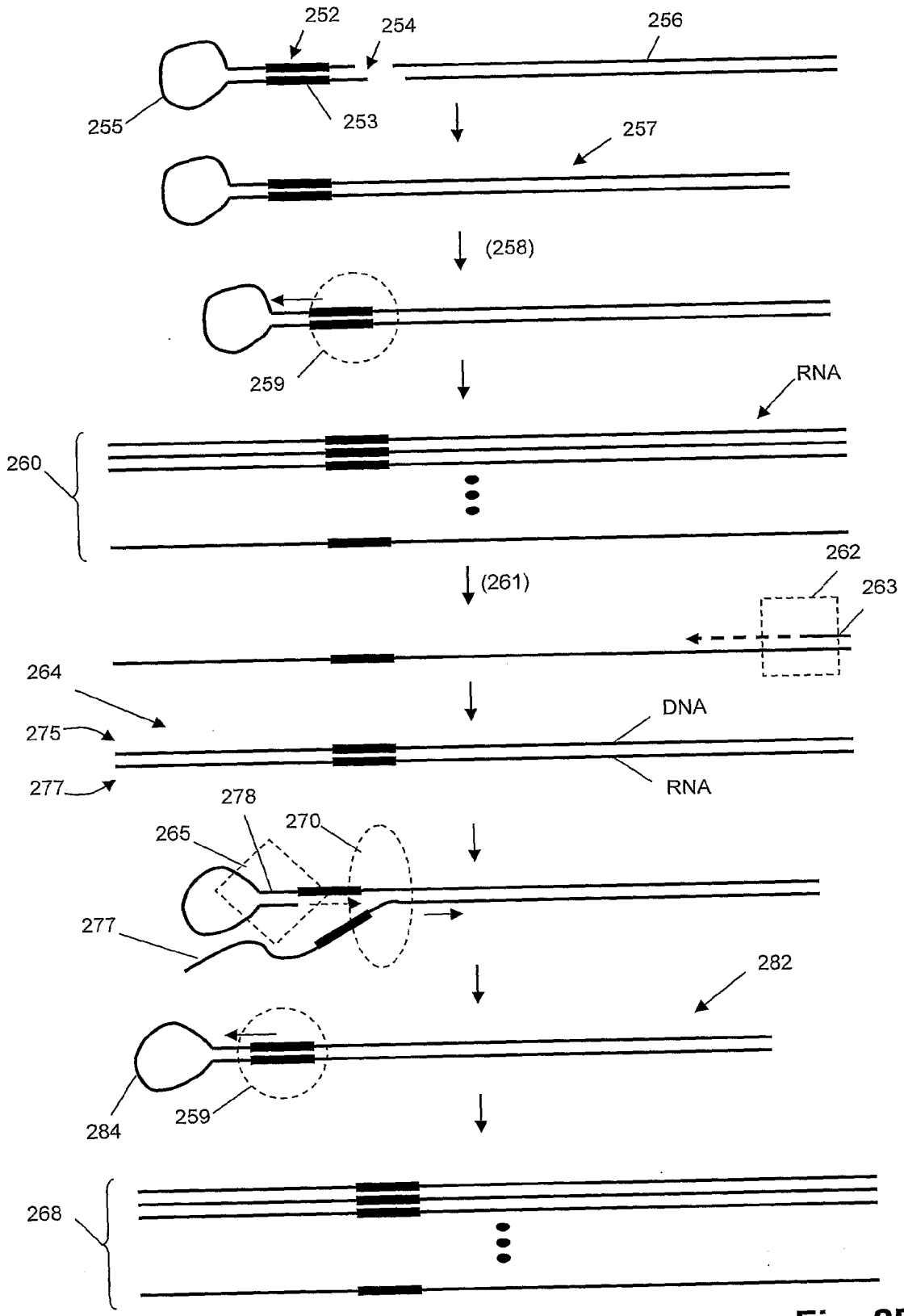

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284
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Fig. 2D

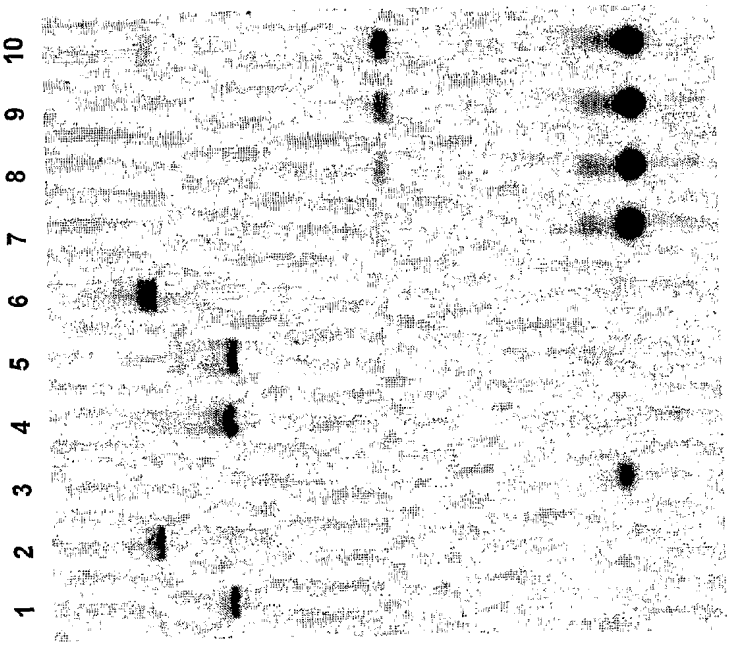


Fig. 3

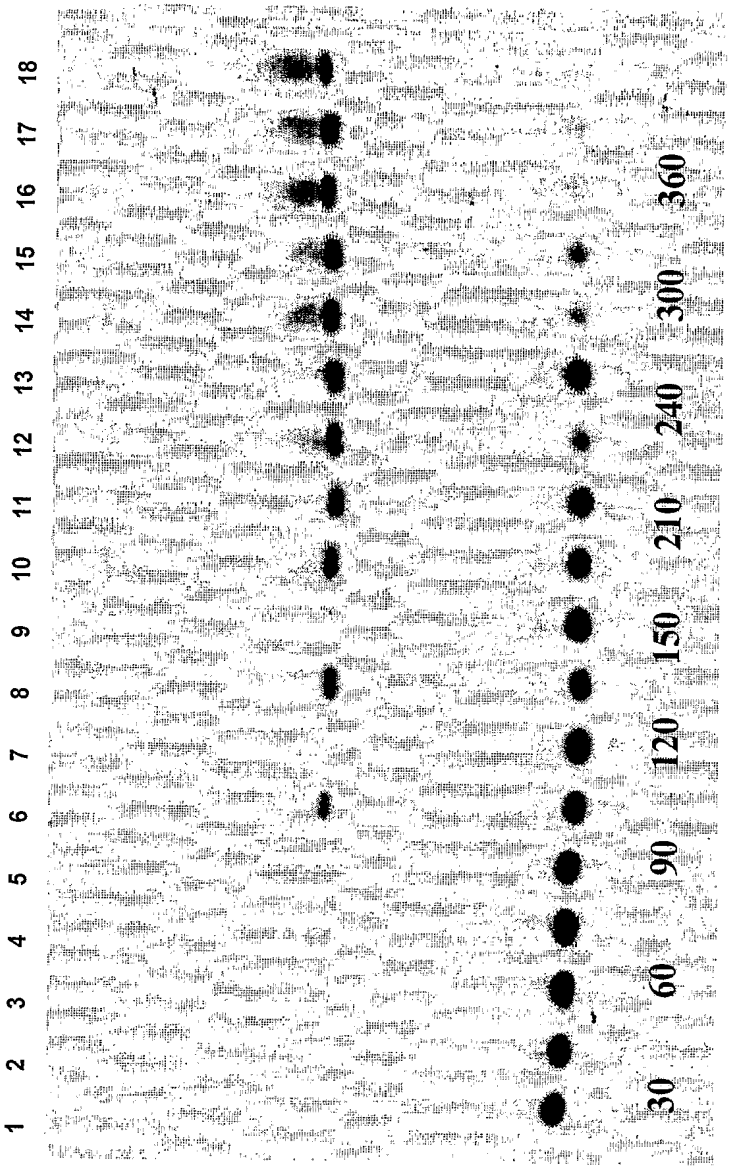

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240
300
360

Fig. 4A

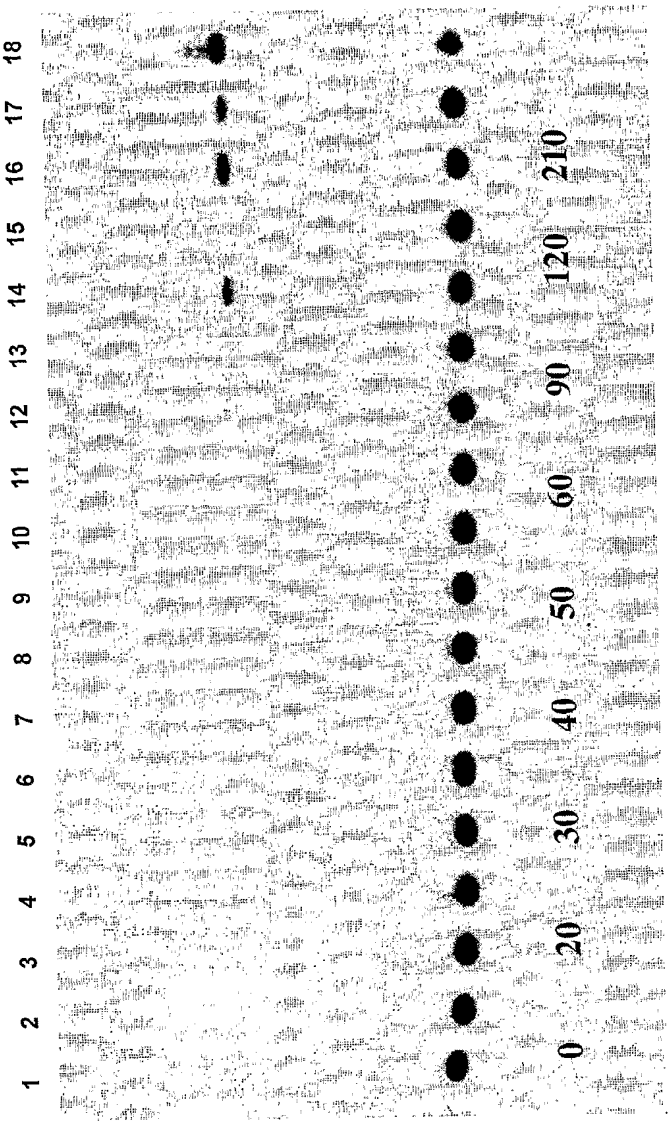


Fig. 4B

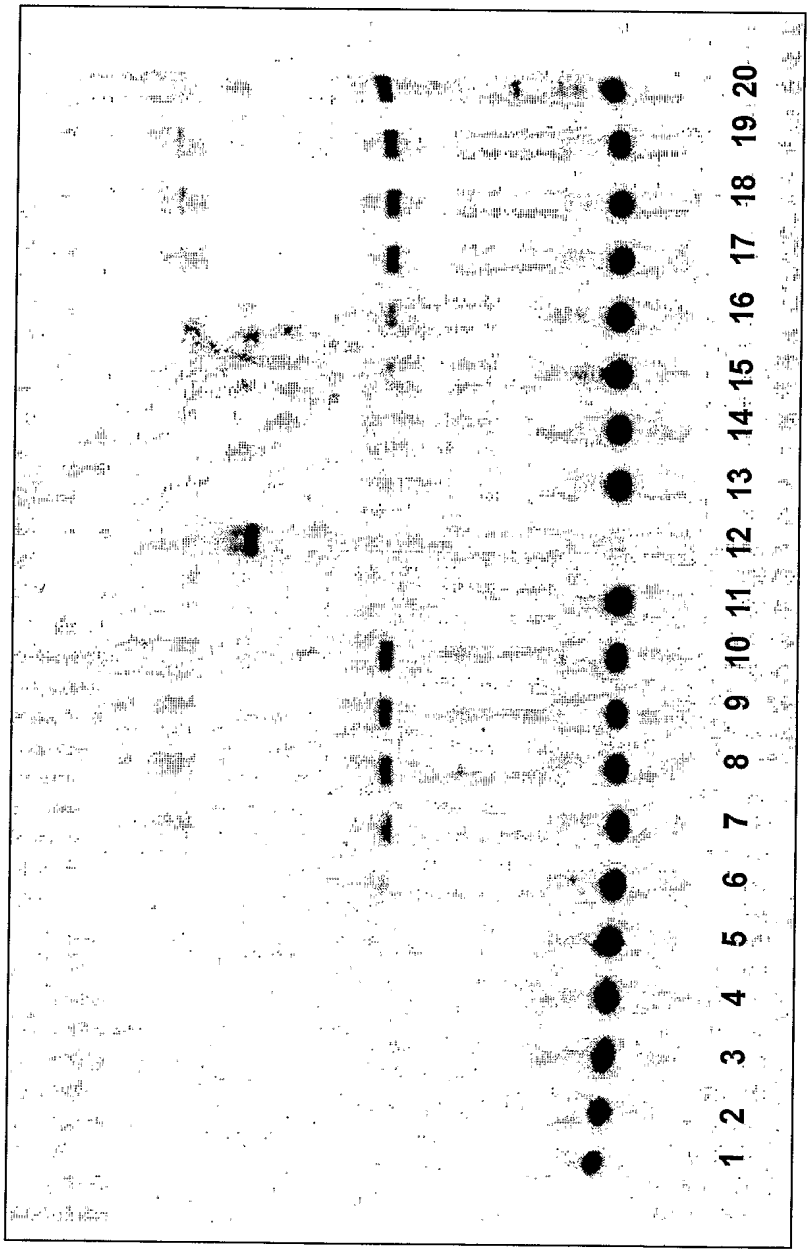


Fig. 5

DNA_{in} (pmol)	Tmax (min)	DNA_{out} (pmol)	Amp Fold
0.001	200	20.8	20800
0.010	150	16.8	1680
0.100	120	12.5	125
1.000	60	10.5	10.5

Table 1

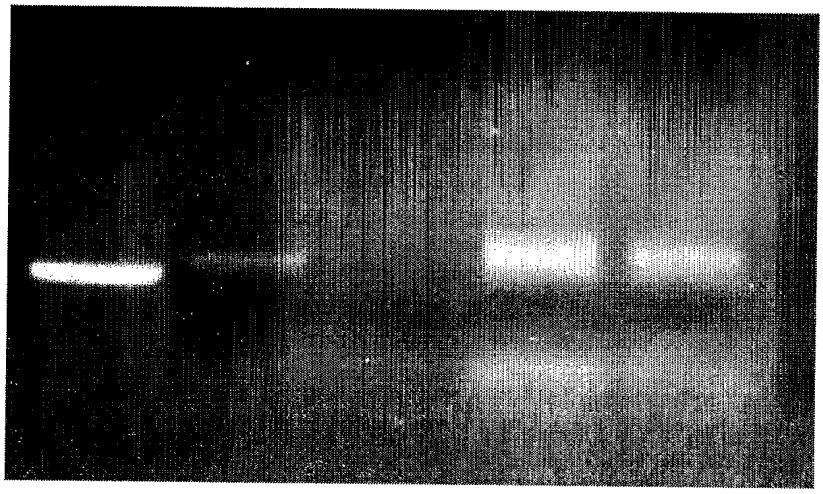


Fig. 6

SEQUENCE LISTING

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Brenner, Sydney
Mikawa, Gi

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<151> 2005-01-25

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gucgauuggc uu 72

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<400> 3

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ggata 65

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gtcgtattaa gcaagg 76

IDT Exhibit 1020.8459

Espacenet

Bibliographic data: WO0120035 (A2) — 2001-03-22

METHODS AND COMPOSITIONS FOR LINEAR ISOTHERMAL AMPLIFICATION OF POLYNUCLEOTIDE SEQUENCES

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Classification: - international: ***C07H19/00; C07H21/02; C07H21/04; C12N15/09; C12Q1/68;*** (IPC1-7): C12Q1/68
- cooperative: **C12Q1/6844**; **C12Q1/6865** more

Application number: WO2000US25104 20000913 Global Dossier

Priority number (s): US19990153604P 19990913 ; US20000175780P 20000112

Also published as: WO0120035 (A3) WO0120035 (A9) AT262595 (T) AU7483500 (A) AU783873 (B2) more

Abstract of WO0120035 (A2)

The present invention provides novel isothermal, single primer linear nucleic acid amplification methods. Methods for amplifying complementary DNA using a composite primer, primer extension, strand displacement, and optionally a termination sequence, are provided. Methods for amplifying sense RNA using a composite primer, primer extension, strand displacement, optionally template switching, a propromoter oligonucleotide and transcription are also provided. The invention further provides compositions and kits for practising said methods, as well as methods which use the amplification products.

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(72) Inventor; and
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(54) Title: METHODS AND COMPOSITIONS FOR LINEAR ISOTHERMAL AMPLIFICATION OF POLYNUCLEOTIDE SEQUENCES

(57) Abstract: The present invention provides novel isothermal, single primer linear nucleic acid amplification methods. Methods for amplifying complementary DNA using a composite primer, primer extension, strand displacement, and optionally a termination sequence, are provided. Methods for amplifying sense RNA using a composite primer, primer extension, strand displacement, optionally template switching, a propromoter oligonucleotide and transcription are also provided. The invention further provides compositions and kits for practising said methods, as well as methods which use the amplification products.

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METHODS AND COMPOSITIONS FOR LINEAR ISOTHERMAL AMPLIFICATION OF POLYNUCLEOTIDE SEQUENCES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the priority benefit of the provisional patent applications U.S. Serial No. 60/153,604, filed September 13, 1999, and U.S. Serial no. 60/175,780, filed January 12, 2000, both of which are incorporated by reference in their entirety.

TECHNICAL FIELD

The invention relates to the field of polynucleotide amplification. More particularly, the invention provides methods, compositions and kits for amplifying (i.e., making multiple copies) target polynucleotide sequences which employ a single RNA/DNA composite primer, with the amplification optionally involving transcription.

BACKGROUND

The developments of methods for nucleic acid amplification and detection of amplification products have advanced the detection, identification, quantification and sequence analyses of nucleic acid sequences in recent years.

Nucleic acid analysis is useful for detection and identification of pathogens, detection of gene alteration leading to defined phenotypes, diagnosis of genetic diseases or the susceptibility to a disease, assessment of gene expression in development, disease and in response to defined stimuli, as well as the various genome projects. Other applications of nucleic acid amplification method are the detection of rare cells, detection of pathogens, and the detection of altered gene expression in malignancy, and the like. Nucleic acid amplification is potentially useful for both qualitative analysis, such as the detection of the presence of defined nucleic acid sequences, and quantification of defined gene sequences. The latter is useful for assessment of and amount of pathogenic sequences as well as the determination of gene multiplication or deletion, as often found in cell transformation from normal to malignant cell type.

The detection of sequence alterations in a nucleic acid sequence is important for the detection of mutant genotypes, as relevant for genetic analysis, the detection of mutations

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leading to drug resistance, pharmacogenomics, etc. Various methods for the detection of specific mutations include allele specific primer extension, allele specific probe ligation, and differential probe hybridization. See, for example, U.S. Patent Nos. 5,888,819; 6,004,744; 5,882,867; 5,710,028; 6,027,889; 6,004,745; and WO US88/02746. Methods for the detection of the presence of sequence alterations in a define nucleic acid sequence, without the specific knowledge of the alteration, were also described. Some of these methods are based on the detection of mismatches formed by hybridization of a test amplification product to a reference amplification product. The presence of mismatches in such hetero-duplexes can be detected by the use of mismatch specific binding proteins, or by chemical or enzymatic cleavage of the mismatch. A method for detection of sequence alteration which is based on the inhibition of branch migration in cruciform four stranded DNA structures was recently described. See, for example, Lishanski, A. et al. *Nucleic Acids Res* 28(9):E42 (2000). Other methods are based on the detection of specific conformations of single stranded amplification products. The secondary structure of a single stranded DNA or RNA is dependent on the specific sequence. Sequence alterations in a test nucleic acid target relative to a reference sequence leads to altered conformation. Altered conformation of a single stranded amplification product can be detected by a change in the electrophoretic mobility of the test amplification product as compared to that of a reference amplification product. Single stranded conformation polymorphism, SSCP, is widely used for the detection of sequence alterations. See, for example, Orita M., et al. *Proc Natl Acad Sci U S A* 86(8):2766-70 (1989); Suzuki, Y. et al. *Oncogene* 5(7):1037-43 (1990); and US. Patent No. 5,871,697. This method is also used in microbial identification that is based on the defined changes in a specific nucleic acid sequence in different strains or species. Mutation detection using the SSCP methods mostly employs DNA amplification products, however, RNA-SSCP methods have also been described. Sequence dependent conformation of a single stranded RNA is well-documented and was shown to lead to a defined electrophoretic mobility pattern. See, for example, Sarkar et al. *Nucleic Acid Research* 20(4):871-878 (1992) and Gasparini et al. *Hum. Genet.* 97:492-495 (1996).

Although detection of the presence of a defined nucleic acid sequence, and its sequence analysis, can be carried out by probe hybridization, the method generally lacks sensitivity when low amounts of the nucleic acid sequence is present in the test sample,

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such as a few molecules. One solution to this obstacle was the development of methods for generation of multiple copies of the defined nucleic acid sequence, which are suitable for further analysis. The methods for generation of multiple copies of a specific nucleic acid sequence are generally defined as target amplification methods. Other methods for increasing the sensitivity of detection of hybridization analysis are based on the generation of multiple products from the hybridized probe, or probes, for example cleavage of the hybridized probe to form multiple products or the ligation of adjacent probes to form a unique hybridization dependent product. Similarly, increased sensitivity of hybridization reaction was achieved by methods for amplification of signals generated by the hybridization event, such as the method based on hybridization of branched DNA probes.

There are many variations of nucleic acid amplification, for example, exponential amplification, linked linear amplification, ligation-based amplification, and transcription-based amplification. An example of exponential nucleic acid amplification method is polymerase chain reaction (PCR) which has been disclosed in numerous publications. See, for example, Mullis et al. *Cold Spring Harbor Symp. Quant. Biol.* 51:263-273 (1986); Mullis K. EP 201,184; Mullis et al. U.S. Patent No. 4,582,788; Erlich et al. EP 50,424, EP 84,796, EP 258,017, EP 237,362; and Saiki R. et al. U.S. Patent No. 4,683,194. Linked linear amplification is disclosed by Wallace et al. in U.S. Patent No. 6,027,923. Examples of ligation-based amplification are the ligation amplification reaction (LAR), disclosed by Wu et al. in *Genomics* 4:560 (1989) and the ligase chain reaction, disclosed in EP Application No. 0320308B1. Various methods of transcription-based amplification are disclosed in U.S. Patent Nos. 5,766,849 and 5,654,142; and also by Kwoh et al. *Proc. Natl. Acad. Sci. U.S.A.* 86:1173 (1989) and Ginergeras et al. WO 88/10315.

The most commonly used target amplification method is the polymerase chain reaction, PCR, which is based on multiple cycles of denaturation, hybridization of two oligonucleotide primers, each to opposite strand of the target strands, and primer extension by a nucleotide polymerase to produce multiple double stranded copies of the target sequence. Many variations of PCR have been described, and the method is being used for amplification of DNA or RNA nucleic acid sequences, sequencing, mutation analysis and others. Thermocycling-based methods that employ a single primer, have also been described. See, for example, U.S. Patent Nos. 5,508,178; 5,595,891; 5,683,879; 5,130,238;

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and 5,679,512. The primer can be a DNA/RNA chimeric primer, as disclosed in U.S. Patent No. 5,744,308. Other methods that are dependent on thermal cycling are the ligase chain reaction (LCR) and the related repair chain reaction (RCR).

Target nucleic acid amplification may be carried out through multiple cycles of incubations at various temperatures, i.e. thermal cycling, or at one temperature (an isothermal process). The discovery of thermostable nucleic acid modifying enzymes has contributed to the fast advances in nucleic acid amplification technology. See Saiki, et al. *Science* 239:487 (1988). Thermostable nucleic acid modifying enzymes, such as DNA and RNA polymerases, ligases, nucleases and the like, are used in both methods dependent on thermal cycling and isothermal amplification methods. Isothermal methods such as strand displacement amplification (SDA) is disclosed by Fraiser et al. in U.S. Patent No. 5,648,211; Cleuziat et al. in U.S. Patent No. 5,824,517; and Walker et al. *Proc. Natl. Acad. Sci. U.S.A.* 89:392-396 (1992). Other isothermal target amplification methods are the transcription-based amplification methods, in which an RNA polymerase promoter sequence is incorporated into primer extension products at an early stage of the amplification (WO 89/01050), and further target sequence, or target complementary sequence, is amplified by transcription steps and digestion of an RNA strand in a DNA/RNA hybrid intermediate product. See, for example, U.S. Patent Nos. 5,169,766 and 4,786,600. These methods include transcription mediated amplification (TMA), self-sustained sequence replication (3SR), Nucleic Acid Sequence Based Amplification (NASBA), and variations there of. See, for example, Guatelli et al. *Proc. Natl. Acad. Sci. U.S.A.* 87:1874-1878 (1990); U.S. Patent Nos. 5,766,849 (TMA); and 5,654,142 (NASBA). Other amplifications methods use template switching oligonucleotides (TSOs) and blocking oligonucleotides. For example, the template switch amplification in which chimeric DNA primer are utilitized is disclosed in U.S. Patent 5,679,512 and by Patel et al. *Proc. Natl. Acad. Sci. U.S.A.* 93:2969-2974 (1996) and blocking oligonucleotides are disclosed by Laney et al. in U.S. Patent No. 5,679,512.

The isothermal target amplification methods do not require a thermocycler, and are thus easier to adapt to common instrumentation platform. However the previously described isothermal target amplification methods have several drawbacks. Amplification according to the SDA methods requires the presence of sites for defined restriction

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enzymes, which limits its applicability. The transcription base amplification methods, such as the NASBA and TMA, on the other hand, are limited by the need for incorporation of the polymerase promoter sequence into the amplification product by a primer, a process prone to result in non-specific amplification. Moreover, the mechanism of amplification of a DNA target by these transcription based amplification methods is not well-established.

Another drawback of the current amplification methods is the potential contamination test samples by amplification products of prior amplification reactions, resulting in non-target specific amplification in samples. This drawback is a well-known problem, which is the result of the power of target amplification technology, and the formation of amplification products, which are substrates for amplification. Various means for decontamination of test samples either at the end of the amplification reaction, or prior to the initiation of target amplification have been described. In additions, method for containment of the test solution by physical means, were also described. All of these solutions are cumbersome and add to the complexity of nucleic acid testing in the common laboratory setting.

Furthermore, amplification methods that use thermocycling process have an added disadvantage of long lag times which are required for the thermocycling block to reach the "target" temperature for each cycle. Consequently, amplification reactions performed using thermocycling processes require a significant amount of time to reach completion.

Therefore, there is a need for improved nucleic acid amplification methods that overcome these drawbacks. The invention provided herein fulfills this need and provides additional benefits.

All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

DISCLOSURE OF THE INVENTION

The invention provides methods and compositions for polynucleotide amplification, as well as applications of the amplification methods.

Accordingly, in one aspect, the invention provides methods for amplifying a polynucleotide sequence complementary to a target polynucleotide sequence comprising: (a) hybridizing a single stranded DNA template comprising the target sequence with a

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composite primer, said composite primer comprising an RNA portion and a 3' DNA portion; (b) optionally hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the template which is 5' with respect hybridization of the composite primer to the template; (c) extending the composite primer with DNA polymerase; (d) cleaving the RNA portion of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer can hybridize to the template and repeat primer extension by strand displacement, whereby multiple copies of the complementary sequence of the target sequence are produced.

In another aspect, the invention provides methods for amplifying a target polynucleotide sequence comprising: (a) hybridizing a single stranded DNA template comprising the target sequence with a composite primer, said composite primer comprising an RNA portion and a 3' DNA portion; (b) hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the template which is 5' with respect hybridization of the composite primer to the template; (c) extending the composite primer with DNA polymerase; (d) cleaving the RNA portion of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer can hybridize to the template and repeat primer extension by strand displacement to produce displaced primer extension product; (e) hybridizing a polynucleotide comprising a propromoter and a region which hybridizes to the displaced primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the displaced primer extension products, whereby multiple copies of the target sequence are produced.

Various embodiments of the composite primer used in the methods of the invention are described herein. For example, in some embodiments, the RNA portion of the composite primer is 5' with respect to the 3' DNA portion. In still other embodiments, the 5' RNA portion is adjacent to the 3' DNA portion. For the methods described herein, one or more composite primers can be used.

Various exemplary embodiments of polynucleotides comprising a termination sequence are also described herein. In some embodiments, the polynucleotide comprising a termination sequence is a template switch oliognucleotide (TSO), which may (but not necessarily) contain one or more modifications to enhance binding to template.

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Accordingly, in some embodiments, the TSO comprises a modification in the region which hybridizes to the template, wherein, under a given set of conditions, the TSO binds more tightly to the region as compared to a TSO without the modification. Examples of suitable modifications are provided herein. In some embodiments, the polynucleotide comprising a termination sequence is a blocking sequence, which, like the TSO, may contain one or more modifications to enhance binding to template. Accordingly, in some embodiments, the blocker sequence comprises a modification in the region which hybridizes to the template, wherein, under a given set of conditions, the blocker binds more tightly to the region as compared to a blocker without the modification. Examples of suitable modifications are provided herein.

The enzymes which may be used in the methods and compositions are described herein. For example, the enzyme that cleaves RNA may be an RNaseH.

In some aspects, a TSO provides propromoter function and also comprises a region (which may or may not be adjacent to the promoter) which hybridizes to the displaced primer extension product. In other embodiments, the polynucleotide comprising the propromoter comprises a region at the 3' end which hybridizes to the displaced primer extension product, whereby DNA polymerase extension of displaced extension product produces a double stranded promoter from which transcription occurs. In some embodiments, the polynucleotide comprising the propromoter is a PTO.

The methods are applicable to amplifying any DNA target, including, for example, genomic DNA and cDNA. One or more steps may be combined and/or performed sequentially (often in any order, as long as the requisite product(s) are able to be formed).

The invention also provides methods which employ (usually, analyze) the products of the amplification methods of the invention, such as sequencing and detection of sequence alteration(s).

Accordingly, in one aspect, the invention provides methods of sequencing a target nucleotide sequence comprising: (a) hybridizing a single stranded DNA template comprising the target sequence with a composite primer, said composite primer comprising an RNA portion and a 3' DNA portion; (b) optionally hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the template which is 5' with respect hybridization of the composite primer to the template; (c) extending the

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composite primer with DNA polymerase and a mixture of dNTPs and dNTP analogs (which may be labelled or unlabelled), such that primer extension is terminated upon incorporation of a dNTP analog which may be labelled or unlabelled; (d) cleaving the RNA portion of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer can hybridize to the template and repeat primer extension by strand displacement, whereby multiple copies of the complementary sequence of the target sequence are produced of varying lengths; (e) analyzing the product of steps (a) through (d) to determine sequence.

In another aspect, the invention provides methods for sequencing a target nucleotide sequence comprising (a) hybridizing a single stranded DNA template comprising the target sequence with a composite primer, said composite primer comprising an RNA portion and a 3' DNA portion; (b) hybridizing the template with a polynucleotide comprising a termination polynucleotide sequence to a region of the template which is 5' with respect to hybridization of the composite primer to the template; (c) extending the composite primer with DNA polymerase; (d) cleaving the RNA portion of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer can hybridize to the template and repeat primer extension by strand displacement to produce displaced primer extension product; (e) hybridizing a polynucleotide comprising a propromoter at the 5' end and a region which hybridizes to the displaced primer extension product under conditions such that transcription occurs from the extension product by RNA polymerase, using a mixture of rNTPs and rNTP analogs (which may be labelled or unlabelled), such that RNA transcripts are produced comprising sequences complementary to the displaced primer extension products, and such that transcription is terminated upon incorporation of an rNTP analog which may be labelled or unlabelled, whereby multiple copies of the target sequence are produced of varying lengths; (f) analyzing the product of steps (a) through (e) to determine sequence.

In some aspects, the invention provides methods of characterizing, or analyzing, sequence of a target. Some aspects are based on the RNA portion of the composite primer and accordingly the results reflect information regarding the corresponding region of the target which, if complementary or of sufficient complementarity, hybridizes to the RNA portion of the composite primer. The amount of product as compared to amount of product

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from performing the same amplification reaction on a reference target sequence indicates the presence or absence of a sequence, which may in turn indicate presence or absence of wildtype, mutant, or allelic variants. Various sequence detection embodiments are described herein. Thus, for example, the invention provides methods of detecting a mutation in a region of a target polynucleotide sequence, comprising conducting an amplification method described herein, wherein the region of the target polynucleotide sequence corresponds to the RNA portion of the composite primer, and wherein a mutation in the target polynucleotide results in detectably fewer amplification products as compared to the amount of amplification products produced from a reference template comprising region corresponding to the RNA portion of the composite primer which does not comprise a mutation. In these embodiments, amplification by strand displacement is decreased as compared to production from a reference template which comprises a region corresponding to the RNA portion of the composite primer which does not contain the mutation (as compared to the RNA portion of the composite primer).

Thus, the invention provides methods of characterizing a sequence of interest in a target polynucleotide, said methods comprising conducting the amplification methods of the invention wherein the sequence of an RNA portion of the composite primer is known, and wherein (a) production of detectably fewer amplification products from the template as compared to the amount of amplification products from a reference template which comprises a region complementary to the RNA portion of the composite primer indicates that the target polynucleotide does not comprise a sequence complementary to the RNA portion of the composite primer and is a sequence variant with respect to the sequence complementary to the RNA portion of the composite primer; or (b) production of detectably more amplification products from the template as compared to the amount of amplification products from a reference template which does not comprise a region which is complementary to the RNA portion of the composite primer indicates that the target polynucleotide comprises a sequence complementary to the RNA portion of the composite primer and is not a sequence variant with respect to the sequence complementary to the RNA portion of the composite primer. In one embodiment, the sequence of an RNA portion of the composite primer comprises a wild type sequence, and the sequence of interest is characterized in determining the presence or absence of the wild type sequence.

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In another embodiment, the sequence of an RNA portion of the composite primer comprises a mutant sequence, and the sequence of interest is characterized in determining the presence or absence of the mutant sequence. In yet another embodiment, the sequence of an RNA portion of the composite primer comprises an allelic sequence, and the sequence of interest in characterized in determining the presence or absence of the allelic sequence.

In other aspects, the invention provides methods of detecting a mutation (or, in some aspects, characterizing a sequence) in a target polynucleotide, comprising (a) conducting an amplification method described herein; and (b) analyzing the amplified products of the method for single stranded conformation, wherein a difference in conformation as compared to a reference single stranded polynucleotide indicates a mutation in the target polynucleotide. In other embodiments, the invention provides methods of detecting a mutation (or, in some aspects, characterizing a sequence) in a target polynucleotide comprising analyzing amplified products of any of the methods described herein for single stranded conformation, wherein a difference in conformation as compared to a reference single stranded polynucleotide indicates a mutation in the target polynucleotide (or, in some aspects, characterizes the target sequence).

In other aspects, the invention provides methods of producing a microarray, comprising (a) conducting an amplification method described herein; and (b) attaching the amplified products onto a solid substrate to make a microarray of the amplified products. In other embodiments, microarrays are produced by attaching amplified products by any of the methods describd herein onto a solid substrate to make a microarray of amplified products.

Any of these applications can use any of the amplification methods (including various components and various embodiments of any of the components) as described herein. For example, the composite primer used may have a 5' RNA portion, which may be adjacent to the 3' DNA portion.

The invention also provides compositions, kits, complexes, reaction mixtures and systems comprising various components (and various combinations of the components) used in the amplification methods described herein. In one aspect, for example, the invention provides compositions comprising a composite primer, said composite primer

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comprising a 3' DNA portion and a 5' RNA portion. In some embodiments, the 5' RNA portion is adjacent to the 3' DNA portion. In still other embodiments, the 5' RNA portion is about 5 to about 20 nucleotides and the 3' DNA portion is about 5 to about 15 nucleotides. In another aspect, the invention provides compositions comprising a TSO, wherein the TSO comprises a modification in the region which hybridizes to the template, wherein, under a given set of conditions, the TSO binds more tightly to the region as compared to a TSO without the modification. In some embodiments, the compositions of the invention comprise any composite primer described herein and a TSO. In still other embodiments, the invention provides compositions comprising any of the composite primers described herein and any blocking sequences described herein, including those containing modifications which enhance binding to template. In other embodiments, the invention provides compositions comprising any of the composite primers described herein and a PTO.

In another aspect, the invention provides compositions comprising any of the complexes (which are generally considered as intermediates with respect to the final amplification products) described herein (see also the figures for schematic depictions of these various complexes). For example, the invention provides compositions comprising a complex of (a) a template strand; and (b) a composite primer, said composite primer comprising a 3' DNA portion and an RNA portion. The RNA portion may be 5' as well as adjacent to the DNA portion. In some embodiments, the complex further comprises a polynucleotide comprising a termination sequence (which may be, for example, a TSO or a blocking sequence). In some embodiments, the complex further comprises a PTO.

In another aspect, the invention provides reaction mixtures (or compositions comprising reaction mixtures) which contain various combinations of components described herein. For example, the invention provides reaction mixtures comprising (a) a polynucleotide template; (b) a composite primer comprising a 3' DNA portion and an RNA portion; and (c) DNA polymerase. As described herein, any of the composite primers may be in the reaction mixture (or a plurality of composite primers), including a composite primer comprises a 5' RNA portion which is adjacent to the 3' DNA portion. The reaction mixture could also further comprise an enzyme which cleaves RNA from an RNA/DNA hybrid, such as RNase H. A reaction mixture of the invention can also comprise any of the

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polynucleotides comprising termination sequences described herein, as well as a polynucleotide comprising a propromoter and a region which hybridizes to displaced primer extension product, and an RNA polymerase. A reaction mixture of the invention can also comprise a PTO.

In another aspect, the invention provides kits for conducting the methods described herein. These kits, in suitable packaging and generally (but not necessarily) containing suitable instructions, contain one or more components used in the amplification methods. For example, the invention provides kits that comprise a composite primer comprising a 3' DNA portion and an RNA portion (which may be 5' and may further be adjacent to the 3' DNA portion). The composite primer in the kits can be any described herein. The kits can contain further components, such as any of (a) a polynucleotide comprising a termination polynucleotide sequence; (b) a polynucleotide comprising a propromoter; (c) any of the enzymes described herein, such as an enzyme which cleaves RNA from an RNA/DNA hybrid (for example, RNaseH); and (d) a polynucleotide comprising a propromoter and a region which hybridizes to displaced primer extension product.

In another aspect, the invention provides systems for effecting the amplification methods described herein. For example, the invention provides systems for amplifying a target polynucleotide sequence or its complement, comprising (a) a composite primer comprising a 3' DNA portion and an RNA portion; (b) DNA polymerase; and (c) an enzyme which cleaves RNA from an RNA/DNA hybrid (such as RNaseH). The composite primer may be any (one or more) described herein, including a composite primer which comprises a 5' RNA portion which is adjacent to the 3' DNA portion.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1A-C is a diagrammatic representation of a single composite primer isothermal linear amplification process.

FIGURE 2A-2C is a diagrammatic representation of an enhanced single primer isothermal linear nucleic acid amplification process involving transcription, using a template switch oligonucleotide sequence.

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FIGURE 3A-3D is a diagrammatic representation of an enhanced single composite primer isothermal linear nucleic acid amplification process involving transcription, using a blocker sequence component.

FIGURE 4 is a diagrammatic representation of detection of a mutation in a template sequence using the single primer isothermal linear amplification process. "X" denotes a mutation on the target DNA at a site complementary to the RNA portion of the composite primer. As shown amplification of the target nucleic acid is blocked when a mutation is present.

FIGURE 5 depicts an ethidium bromide stained PAGE gel of linear isothermal amplification products of a synthetic DNA target.

FIGURE 6 depicts an autoradiogram of PAGE analysis of hybridization of DNA amplification products to a specific probe.

FIGURE 7 depicts an ethidium bromide stained PAGE gel comparing the efficiency of ssRNA transcription products generated from overlap extension.

FIGURE 8 depicts an ethidium bromide stained PAGE gel comparison of isothermal linear amplification with and without a 3'-blocked PTO.

FIGURE 9 depicts an autoradiogram of probe hybridized to amplification products generated by isothermal linear amplification of a J gene sequence from *E. coli* genomic DNA.

FIGURE 10 depicts an ethidium bromide stained PAGE gel of linear isothermal amplification RNA products generated using three different designs of the composite primer.

MODES FOR CARRYING OUT THE INVENTION

The invention provides methods, compositions and kits for amplifying polynucleotide sequences. The methods generally comprise using an RNA/DNA composite primer, optionally a termination sequence, and, in embodiments in which transcription is used, a propromoter oliognucleotide sequence.

As a general summary, the amplification methods work as follows: a composite RNA/DNA primer forms the basis for replication of target sequence. In some

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embodiments, a termination sequence provides the basis for an endpoint for the replication by either diverting or blocking further replication along the target strand. As described below, in some embodiments, the polynucleotide comprising a termination sequence is a template switch oligonucleotide (TSO), which contains sequences that are not of sufficient complementarity to hybridize to the template strand (in addition to sequences which are of sufficient complementary to hybridize); in other embodiments, the termination sequence comprises primarily sequences that are of sufficient complementarity to hybridize to the template strand. DNA polymerase effects copying of the target sequence from the primer. An enzyme which cleaves RNA from an RNA/DNA hybrid (such as RNaseH) cleaves (removes) RNA sequence from the hybrid, leaving sequence on the template strand available for binding by another composite primer. Another strand is produced by DNA polymerase, which displaces the previously replicated strand, resulting in displaced extension product. Optionally, a polynucleotide comprising a propromoter and a region which hybridizes to the displaced primer extension product (which can be, for example, a template switch oligonucleotide or propromoter template oligonucleotide), which contains sequences of sufficient complementarity to hybridize to the 3' end of the displacement extension product, binds to the displaced primer extension product. The promoter drives transcription (via DNA-dependent RNA polymerase) to produce sense RNA products.

Accordingly, the invention provides methods of producing at least one copy of a target polynucleotide sequence (generally, methods of amplifying target polynucleotide sequence) comprising combining and reacting the following: (a) a single-stranded target polynucleotide comprising a target sequence; (b) a composite primer comprising an RNA portion and a 3' DNA portion; (c) a DNA polymerase; (d) deoxyribonucleoside triphosphates or suitable analogs; (e) an enyzme, such as RNaseH, which cleaves RNA from an RNA/DNA duplex; and (f) generally, but optionally, a polynucleotide comprising a termination sequence, such as any of those described herein, which comprises a portion (or region) which hybridizes to the template polynucleotide. A termination sequence is used if transcription-based amplification (see below) is also used. The combination is subjected to suitable conditions such that (a) the composite primer (and, optionally, a polynucleotide comprising a termination sequence) hybridizes to the template; (b) primer extension occurs from the composite primer, to form a duplex; (c) RNaseH cleaves RNA of the composite

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primer from the RNA/DNA duplex; (d) another composite primer hybridizes to the template, and another round of primer extension (mediated by DNA polymerase) occurs, displacing the strand already copied from the template.

Optionally, the following is also included in the amplification reaction (either at the same time as those components listed above or added separately): (e) a polynucleotide comprising a propromoter sequence (which can be in any of a number of forms, as described herein) and a region which hybridizes to the displaced primer extension product; (f) ribonucleoside triphosphates or suitable analogs; and (g) RNA polymerase, under conditions such that transcription of the displaced strand can occur. Details regarding the various components of the methods of the present invention are provided below.

In some embodiments, the invention provides methods of sequencing nucleic acids (DNA or RNA). For the sequencing methods, the appropriate dNTPs (or, when embodiments which rely on transcription-based amplifications are used, appropriate rNTPs), which may be labelled or unlabelled, are used. Accordingly, the invention provides methods of sequencing a target nucleotide sequence comprising the methods described above, wherein dNTPs and dNTP analogs which are primer elongation terminators, which may be labelled or unlabelled, and/or rNTPs and rNTP analogs, which are primer elongation terminators, which may be labelled or unlabelled, are used, and the amplification product is analyzed for sequence information, as described below.

In other embodiments, the invention provides methods of detecting nucleic acid sequence mutations and/or characterizing target sequence(s). In one embodiment, the presence or absence of a mutation in a target polynucleotide is detected based on the ability to amplify the target polynucleotide using a composite primer whose RNA portion either contains or lacks the mutated sequence using the methods of the invention. In another embodiment, the amplified products are used for detection of mutations by hybridization with specific probes. In yet another embodiment, the amplified products are used to detect and/or identify single strand conformation polymorphisms in a target polynucleotide.

In yet other embodiments, the invention provides methods for generating microarrays of nucleic acids (DNA or RNA) using the amplified products of the linear or enhanced linear nucleic acid amplification methods of the present invention.

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Other methods which use the amplified products described herein are provided below.

Advantages of the amplification methods of the invention

The amplification methods of the invention provide several significant advantages over other methods of nucleic acid amplification. The formation of primed template, primer extension and displacement of the previously generated extension product is dependent on the cleavage of the RNA portion of the hybridized primer by a ribonuclease activity. Thus, the primer extension product is lacking the 5'-most portion of the primer. Consequently, the RNA transcription product generated from the complex of the extension product and the template switch oligonucleotide, or the promoter template oligonucleotide, does not contain at its 3' end the sequence complementary to this portion of the primer. Thus, the amplification products are not capable of hybridizing to the primer for productive amplification, making the amplification methods of the invention resistant to non-specific amplification due to contamination with products generated by prior amplifications reactions. This feature clearly distinguishes it from other known target amplification methods, such as PCR, NASBA and the like, and renders the methods of the invention suitable for open tube platforms commonly used in clinical laboratories, high throughput testing sites, and the like.

The unique requirement of cleavage of the RNA portion of the composite primer in the hybridized and extended form, by a ribonuclease such as RNase H, for further progression of the amplification of the target nucleic acid sequence, results in the exclusive amplification of DNA target. Thus, it is possible to use the methods of the invention for amplification of genomic DNA targets in the presence of excess mRNA. This feature is useful for accurate quantification of gene dosage. When the test target nucleic acid sequence is RNA, the target is first transcribed to produce cDNA which can be amplified using the methods of the invention.

The methods of the invention also provide for amplification of target nucleic acids with high accuracy with respect to the template. Each amplification product is a direct copy of the target sequence in the input template DNA (in the linear amplification

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methods), or the input template DNA and the primer extension products of the input template DNA (in the enhanced linear amplification methods).

The methods of the invention do not require thermocycling in that amplification can be performed isothermally. This feature provides numerous advantages, including facilitating automation and adaptation for high throughput amplification and/or analysis of nucleic acids. For example, sequencing methods based on the amplification methods of the invention are simplified by the ability to perform the reactions isothermally. Other methods that have been reported require thermal cycling for the separation of primer extension products from the target sequence. The isothermal reaction is faster than that afforded by thermal cycling and is suitable for performing sequencing of a target nucleic acid in miniaturized devices.

Another advantage of the methods of the invention is that only a single primer is required. A single primer is utilized to provide unidirectional primer extension that results in amplification of template nucleic acid. This obviates the numerous drawbacks associated with having to use primer pairs, for example cost of designing and making two sets of primers, the need to have prior knowledge of an additional sequence region within the template nucleic acid, and the increased probability that amplified products are the result of non-specific priming.

The linear isothermal amplification methods of the invention are also suitable for use for detection of nucleic acid targets, quantification of defined nucleic acid sequences, and the production of probes for defined nucleic acid sequences. The methods of the invention are useful for qualitative detection of a nucleic acid sequence, quantitative determination of the amount of the target nucleic acid sequence, detection of the presence of defined sequence alterations, as needed for genotyping, and sequencing. The products of the amplification according to the methods of the invention are single stranded and are readily detectable by various known nucleic acid detection methods.

The methods of the invention are further useful for multiplex analysis of nucleic acid sequences. That is to say, various target sequences may be amplified simultaneously in a single reaction mixture. The various target sequences may be part of a single genomic DNA, or may represent specific sequences of various nucleic acid targets, which may be present in a single test sample. For example, the methods of the invention are useful for the

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detection of the presence of various pathogens in a single biological sample. Similarly, the determination of various polymorphic sites in a single genomic DNA sample can be determined simultaneously in a single reaction.

It is understood that, with respect to all embodiments described herein, as generally "comprising" components or aspects, the invention also includes embodiments which "consist essentially of" these components or aspects. The invention also includes embodiments which "consist of" these components or aspect. This applies to all embodiments described herein.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994).

Primers, oligonucleotides and polynucleotides employed in the present invention can be generated using standard techniques known in the art.

Definitions

A "target sequence," as used herein, is a polynucleotide sequence of interest, for which amplification is desired. The target sequence may be known or not known, in terms of its actual sequence. Generally, a "template," as used herein, is a polynucleotide that contains the target nucleotide sequence. In some instances, the terms "target sequence," "template DNA," "template polynucleotide," "target nucleic acid," "target polynucleotide," and variations thereof, are used interchangeably.

"Amplification," as used herein, generally refers to the process of producing multiple copies of a desired sequence. "Multiple copies" mean at least 2 copies. A "copy"

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does not necessarily mean perfect sequence complementarity or identity to the template sequence. For example, copies can include nucleotide analogs such as deoxyinosine, intentional sequence alterations (such as sequence alterations introduced through a primer comprising a sequence that is hybridizable, but not complementary, to the template), and/or sequence errors that occur during amplification.

"Polynucleotide," or "nucleic acid," as used interchangeably herein, refer to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. If present, modification to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications include, for example, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, cabamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, ply-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide(s). Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping groups moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'--O-methyl-, 2'-O-allyl, 2'-fluoro- or 2'-azido-ribose,

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carbocyclic sugar analogs, α-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs and abasic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S("thioate"), P(S)S ("dithioate"), "(O)NR_2 ("amidate"), P(O)R, P(O)OR', CO or CH_2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

"Oligonucleotide," as used herein, generally refers to short, generally single stranded, generally synthetic polynucleotides that are generally, but not necessarily, less than about 200 nucleotides in length. Oligonucleotides in the present invention include the composite primer, TSO, PTO and blocker sequence. The terms "oligonucleotide" and "polynucleotide" are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides.

A "primer" is generally a short single stranded polynucleotide, generally with a free 3'-OH group, that binds to a target potentially present in a sample of interest by hybridizing with a target sequence, and thereafter promotes polymerization of a polynucleotide complementary to the target.

A "termination polynucleotide sequence" or "termination sequence," as used interchangeably herein, is a polynucleotide sequence which effects cessation of DNA replication by DNA polymerase with respect to the template comprising the target sequence. A termination sequence comprises a portion (or region) that generally hybridizes to the template at a location 5' to the termination point (site). The hybridizable portion may or may not encompass the entire termination sequence. Examples of suitable termination polynucleotide sequences (such as blocker sequences and TSOs) are provided herein.

"Blocker sequence," or "blocking sequence" as used interchangeably herein, is an example of a termination sequence, and refers to an oligonucleotide that binds, generally with high affinity, to the template nucleic acid at a location 5' to the termination site and

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effects cessation of DNA replication by DNA polymerase with respect to the template comprising the target sequence. Its 3' end may or may not be blocked for extension by DNA polymerase.

"Termination site," or "termination point," as used interchangeably herein, refers to the site, point or region of the template that is last replicated by the DNA polymerase before termination of polymerization (generally, primer extension) or template switch. For example, with respect to a TSO, it is the position or region in the target sequence that is complementary to the 3' end of the primer extension product prior to switching template from the template polynucleotide to the unhybridized portion of the TSO.

"Protopromoter sequence," and "propromoter sequence," as used herein, refer to a single-stranded DNA sequence region which, in double-stranded form is capable of mediating RNA transcription. In some contexts, "protopromoter sequence," "protopromoter," "propromoter sequence," "propromoter," "promoter sequence," and "promoter" are used interchangeably.

"Template switch oligonucleotide (TSO)," as used herein, refers to an oligonucleotide that comprises a portion (or region) that is hybridizable to a template at a location 5' to the termination site of primer extension and that is capable of effecting a template switch in the process of primer extension by a DNA polymerase. TSOs are generally known in the art. "Template switch" refers to a change in template nucleic acid, generally from the target nucleic acid to the unhybridized portion of a TSO, during the course of a single round of primer extension.

"Propromoter template oligonucleotide (PTO)," as used herein, refers to an oligonucleotide that comprises a propromoter sequence and a portion, generally a 3' portion, that is hybridizable to the 3' region of a primer extension product. The propromoter sequence and the hybridizable portion may be the same, distinct or overlapping nucleotides of an oligonucleotide.

A first sequence which "corresponds" to second sequence, such as an RNA portion of a composite primer, means that the first sequence has significant sequence identity with respect to the second sequence. This term is generally used in the context of detecting mutations, or characterizing sequences of a target.

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To "inhibit" is to decrease or reduce an activity, function, and/or amount as compared to a reference.

A "complex" is an assembly of components. A complex may or may not be stable and may be directly or indirectly detected. For example, as is described herein, given certain components of a reaction, and the type of product(s) of the reaction, existence of a complex can be inferred. For purposes of this invention, a complex is generally an intermediate with respect to the final amplification product(s).

A "portion" or "region," used interchangeably herein, of a polynucleotide or oligonucleotide is a contiguous sequence of 2 or more bases. In other embodiments, a region or portion is at least about any of 3, 5, 10, 15, 20, 25 contiguous nucleotides.

A region, portion, or sequence which is "adjacent" to another sequence directly abuts that region, portion, or sequence. For example, an RNA portion which is adjacent to a 5' DNA portion of a composite primer directly abuts that region. For an illustration of this example, see Figure 1A-C.

A "reaction mixture" is an assemblage of components, which, under suitable conditions, react to form a complex (which may be an intermediate) and/or a product(s).

"A", "an" and "the", and the like, unless otherwise indicated include plural forms.

"Comprising" means including.

Conditions that "allow" an event to occur or conditions that are "suitable" for an event to occur, such as hybridization, strand extension, and the like, or "suitable" conditions are conditions that do not prevent such events from occurring. Thus, these conditions permit, enhance, facilitate, and/or are conducive to the event. Such conditions, known in the art and described herein, depend upon, for example, the nature of the nucleotide sequence, temperature, and buffer conditions. These conditions also depend on what event is desired, such as hybridization, cleavage, strand extension or transcription.

Sequence "mutation," as used herein, refers to any sequence alteration in a sequence of interest in comparison to a reference sequence. A reference sequence can be a wild type sequence or a sequence to which one wishes to compare a sequence of interest. A sequence mutation includes single nucleotide changes, or alterations of more than one nucleotide in a sequence, due to mechanisms such as substitution, deletion or insertion. Single nucleotide polymorphism (SNP) is also a sequence mutation as used herein.

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"Single stranded conformation polymorphism," and "SSCP," as used herein, generally refer to the specific conformation of a single stranded nucleic acid as is affected by its specific nucleic acid sequence. Alteration of the sequence of the single stranded polynucleotide, such as single nucleotide substitution, deletions or insertions, result in change, or polymorphism, of the conformation of the single stranded polynucleotide. The conformation of the polynucleotide is generally detectable, identifiable and/or distinguishable using methods known in the art, such as electrophoretic mobility as measured by gel electrophoresis, capillary electrophoresis, and/or susceptibility to endonuclease digestion.

"Microarray" and "array," as used interchangeably herein, refer to an arrangement of a collection of nucleotide sequences in a centralized location. Arrays can be on a solid substrate, such as a glass slide, or on a semi-solid substrate, such as nitrocellulose membrane. The nucleotide sequences can be DNA, RNA, or any permutations thereof.

The term "3'" generally refers to a region or position in a polynucleotide or oligonucleotide 3' (downstream) from another region or position in the same polynucleotide or oligonucleotide.

The term "5'" generally refers to a region or position in a polynucleotide or oligonucleotide 5' (upstream) from another region or position in the same polynucleotide or oligonucleotide.

The term "3'-DNA portion," "3'-DNA region," "3'-RNA portion," and "3'-RNA region," refer to the portion or region of a polynucleotide or oligonucleotide located towards the 3' end of the polynucleotide or oligonucleotide, and may or may not include the 3' most nucleotide(s) or moieties attached to the 3' most nucleotide of the same polynucleotide or oligonucleotide. The 3' most nucleotide(s) can be preferably from about 1 to about 20, more preferably from about 3 to about 18, even more preferably from about 5 to about 15 nucleotides.

The term "5'-DNA portion," "5'-DNA region," "5'-RNA portion," and "5'-RNA region," refer to the portion or region of a polynucleotide or oligonucleotide located towards the 5' end of the polynucleotide or oligonucleotide, and may or may not include the 5' most nucleotide(s) or moieties attached to the 5' most nucleotide of the same polynucleotide or oligonucleotide. The 5' most nucleotide(s) can be preferably from about

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1 to about 20, more preferably from about 3 to about 18, even more preferably from about 5 to about 15 nucleotides.

"Detection" includes any means of detecting, including direct and indirect detection. For example, "detectably fewer" products may be observed directly or indirectly, and the term indicates any reduction (including no products). Similarly, "detectably more" product means any increase, whether observed directly or indirectly.

Components and reaction conditions used in the methods of the invention

Template nucleic acid

The nucleic acid (NA) target to be amplified includes nucleic acids from any source in purified or unpurified form, which can be DNA (dsDNA and ssDNA) or RNA, including tRNA, mRNA, rRNA, 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, and fragments thereof. Otaining and purifying nucleic acids use standard techniques in the art. Amplification of an RNA target will require initial cDNA synthesis, as known in the art. Amplification of a DNA-RNA hybrid would require denaturation of the hybrid to obtain a ssDNA, or denaturation followed by reverse transcription to obtain a cDNA. The target nucleic acid can be only a minor fraction of a complex mixture such as a biological sample and can be obtained from various biological material by procedures well known in the art.

The initial step of the amplification of a target nucleic acid sequence is rendering the target single stranded. If the target nucleic acid is a double stranded (ds) DNA, the initial step is target denaturation. The denaturation step may be thermal denaturation or any other method known in the art, such as alkali treatment. If the target is RNA, the initial step may be the synthesis of a single stranded cDNA. Techniques for the synthesis of cDNA from RNA are known in the art.

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Composite primer

The amplification methods of the invention employ a single composite primer that is composed of RNA and DNA portions. The composite design of the primer is critical for subsequent displacement of the primer extension product by binding of a new (additional) composite primer and the extension of the new primer by the polymerase. In addition, cleavage of the RNA portion of the primer extension product leads to generation of amplification product which is not a substrate for amplification by the composite primer, as described below.

Composite primers for use in the methods and compositions of the present invention comprise at least one RNA portion that is capable of (a) binding (hybridizing) to a sequence on the target nucleic acid (template) independent of hybridization of the DNA portion(s) to a sequence on the target nucleic acid; and (b) being cleaved with a ribonuclease when hybridized to the target DNA. The composite primers bind to the target nucleic to form a partial heteroduplex in which only the RNA portion of the primer is cleaved upon contact with a ribonuclease such as RNase H, while the target strand remains intact, thus enabling annealing of another composite primer.

The composite primers also comprise a 3' DNA portion that is capable of hybridization to a sequence on the target nucleic acid (template) such that its hybridization to the target sequence (template) is favored over that of the nucleic acid strand that is displaced from the target nucleic acid by the DNA polymerase. Such primers can be rationally designed based on well known factors that influence nucleic acid binding affinity, such as sequence length and/or identity, as well as hybridization conditions. In a preferred embodiment, hybridization of the 3' DNA portion of the composite primer to its complementary sequence in the target nucleic acid is favored over the hybridization of the homologous sequence in the 5' end of the displaced strand to the target nucleic acid.

Generation of primers suitable for extension by polymerization is well known in the art, such as described in PCT Pub. No. WO99/42618 (and references cited therein). The composite primer comprises a combination of RNA and DNA (see definition above), with the 3'-end nucleotide being a nucleotide suitable for nucleic acid extension. The 3'-end nucleotide can be any nucleotide or analog that when present in a primer, is extendable by a DNA polymerase. Generally, the 3'-end nucleotide has a 3'-OH. Suitable primers include

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those that comprise at least one portion of RNA and at least one portion of DNA. As shown in Example 5 (showing the relative performance of the various primers used for amplification of E. coli J gene) for one gene, composite primers can comprise a 5'-RNA portion and a 3'-DNA portion (in which the RNA portion is adjacent to the 3'-DNA portion); or 5'- and 3'-DNA portions with an intervening RNA portion. Accordingly, in one embodiment, the composite primer comprises a 5' RNA portion and a 3'-DNA portion, preferably wherein the RNA portion is adjacent to the 3'-DNA portion. In another embodiment, the composite primer comprises 5'- and 3'-DNA portions with at least one intervening RNA portion (i.e., an RNA portion between the two DNA portions). In yet another embodiment, the composite primer of the present invention comprises a 3'-DNA portion and at least one intervening RNA portion (i.e., an RNA portion between DNA portions).

The length of an RNA portion in a composite primer comprising a 3'-DNA portion and an RNA portion can be preferably from about 1 to about 25, more preferably from about 3 to about 20, even more preferably from about 4 to about 15, and most preferably from about 5 to about 10 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and an RNA portion, an RNA portion can be at least about any of 1, 3, 4, 5 nucleotides, with an upper limit of about any of 10, 15, 20, 25, 30 nucleotides.

The length of the 5'-RNA portion in a composite primer comprising a 5'-RNA portion and a 3'-DNA portion can be preferably from about 3 to about 25 nucleotides, more preferably from about 5 to about 20 nucleotides, even more preferably from about 7 to about 18 nucleotides, preferably from about 8 to about 17 nucleotides, and most preferably from about 10 to about 15 nucleotides. In other embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, the 5'-RNA portion can be at least about any of 3, 5, 7, 8, 10 nucleotides, with an upper limit of about any of 15, 17, 18, 20 nucleotides.

In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion further comprising non-5'-RNA portion(s), a non-5'-RNA portion can be preferably from about 1 to about 7 nucleotides, more preferably from about 2 to about 6 nucleotides, and most preferably from about 3 to about 5 nucleotides. In certain

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embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion further comprising non-5'-RNA portion(s), a non-5'-RNA portion can be at least about any of 1, 2, 3, 5, with an upper limit of about any of 5, 6, 7, 10 nucleotides.

In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the length of the 5'-RNA portion can be preferably from about 3 to about 25 nucleotides, more preferably from about 5 to about 20 nucleotides, even more preferably from about 7 to about 18 nucleotides, preferably from about 8 to about 17 nucleotides, and most preferably from about 10 to about 15 nucleotides. In certain embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the 5'-RNA portion can be at least about any of 3, 5, 7, 8, 10 nucleotides, with an upper limit of about any of 15, 17, 18, 20 or nucleotides.

The length of an intervening RNA portion in a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion can be preferably from about 1 to about 7 nucleotides, more preferably from about 2 to about 6 nucleotides, and most preferably from about 3 to about 5 nucleotides. In some embodiments of a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion, an intervening RNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 5, 6, 7, 10 nucleotides. The length of an intervening RNA portion in a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion can be preferably from about 1 to about 7 nucleotides, more preferably from about 2 to about 6 nucleotides, and most preferably from about 3 to about 5 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, an intervening RNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 5, 6, 7, 10 nucleotides. In a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, further comprising a 5'-RNA portion, the 5'-RNA portion can be preferably from about 3 to about 25 nucleotides, more preferably from about 5 to about 20 nucleotides, even more preferably from about 7 to about 18 nucleotides, preferably from about 8 to about 17 nucleotides, and most preferably from about 10 to about 15 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion,

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further comprising a 5'-RNA portion, the 5'-RNA portion can be at least about any of 3, 5, 7, 8, 10 nucleotides, with an upper limit of about any of 15, 17, 18, 20 nucleotides.

The length of the 3'-DNA portion in a composite primer comprising a 3'-DNA portion and an RNA portion can be preferably from about 1 to about 20, more preferably from about 3 to about 18, even more preferably from about 5 to about 15, and most preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and an RNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides.

The length of the 3'-DNA portion in a composite primer comprising a 5'-RNA portion and a 3'-DNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, the 3' DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides.

In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, further comprising non-3'-DNA portion(s), a non-3'-DNA portion can be preferably from about 1 to about 10 nucleotides, more preferably from about 2 to about 8 nucleotides, and most preferably from about 3 to about 6 nucleotides. In some embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion, further comprising non-3'-DNA portion(s), a non-3'-DNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 6, 8, 10, 12 nucleotides.

In embodiments of a composite primer comprising a 5'-RNA portion and a 3'-DNA portion in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the length of the 3'-DNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In certain embodiments of the primer comprising a 5'-RNA portion and a 3'-DNA portion in which the 5'-RNA portion is adjacent to the 3'-DNA portion, the 3'-DNA portion can be at least

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about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides.

The length of a non-3'-DNA portion in a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion can be preferably from about 1 to about 10 nucleotides, more preferably from about 2 to about 8 nucleotides, and most preferably from about 3 to about 6 nucleotides. In some embodiments of a primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion, a non-3'-DNA portion can be at least about any of 1, 2, 3, 5 nucleotides, with an upper limit of about any of 6, 8, 10, 12 nucleotides.

The length of the 3'-DNA portion in a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising 5'- and 3'-DNA portions with at least one intervening RNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides.

The length of a non-3'-DNA portion (i.e., any DNA portion other than the 3'-DNA portion) in a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion can be preferably from about 1 to about 10 nucleotides, more preferably from about 2 to about 8 nucleotides, and most preferably from about 3 to about 6 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, a non-3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 6, 8, 10, 12 nucleotides. The length of the 3'-DNA portion in a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion can be preferably from about 1 to about 20 nucleotides, more preferably from about 3 to about 18 nucleotides, even more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 12 nucleotides. In some embodiments of a composite primer comprising a 3'-DNA portion and at least one intervening RNA portion, the 3'-DNA portion can be at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 12, 15, 18, 20, 22 nucleotides. It is

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understood that the lengths for the various portions can be greater or less, as appropriate under the reaction conditions of the methods of this invention.

In some embodiments, the 5'-DNA portion of a composite primer includes the 5'-most nucleotide of the primer. In some embodiments, the 5'-RNA portion of a composite primer includes the 5' most nucleotide of the primer. In other embodiments, the 3'-DNA portion of a composite primer includes the 3' most nucleotide of the primer. In other embodiments, the 3'-DNA portion is adjacent to the 5'-RNA portion and includes the 3' most nucleotide of the primer (and the 5'-RNA portion includes the 5' most nucleotide of the primer).

The total length of the composite primer can be preferably from about 10 to about 40 nucleotides, more preferably from about 15 to about 30 nucleotides, and most preferably from about 20 to about 25 nucleotides. In some embodiments, the length can be at least about any of 10, 15, 20, 25 nucleotides, with an upper limit of about any of 25, 30, 40, 50 nucleotides. It is understood that the length can be greater or less, as appropriate under the reaction conditions of the methods of this invention.

To achieve hybridization (which, as is well known and understood in the art, depends on other factors such as, for example, ionic strength and temperature), composite primers for use in the methods and compositions of the present invention are preferably of at least about 60%, more preferably at least about 75%, even more preferably at least about 90%, and most preferably at least about 95% complementarity to the target nucleic acid. The individual DNA and RNA portions of the composite primers are preferably of at least about 60%, more preferably at least about 75%, even more preferably at least about 90%, and most preferably at least about 95% complementarity to the target nucleic acid.

As described herein, one or more composite primers may be used in an amplification reaction.

A polynucleotide comprising a termination polynucleotide sequence

In some embodiments of the methods of the present invention, especially if transcription-based amplification is used, a polynucleotide comprising a termination sequence is included, examples of which are provided below.

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Template switch oligonucleotide

A second oligonucleotide that can be used in the amplification methods of the invention is a termination switch oligonucleotide (TSO). In one embodiment, the TSO functions as a termination sequence. In another embodiment, the TSO functions as a termination sequence and provides a propromoter sequence.

Previously described amplification methods based on template switch oligonucleotide were restricted in the concentration of this oligonucleotide due to inhibition of hybridization of the second primer, or the second hybridization step of the same primer when the method is designed to utilize a single primer species. The methods of the invention are free of this limitation. In contrast to previously described methods using TSOs, the template switch oligonucleotide can be used at high concentration for amplification according to the methods of the present invention. This feature ensures efficient hybridization of the oligonucleotide to the target strand, and maximizes the yield of the tri molecular complex, the substrate for primer extension and template switch. An additional attribute of this feature is the efficient hybridization of the displaced primer extension product to the template switch oligonucleotide to form a substrate for the RNA polymerase, as described.

A TSO comprises a 3' portion that can hybridize to the target and a 5' portion which is designed for strand switch during polymerization (see Figure 2A-C). Design of a TSO that would effect strand switch is known in the art, such as was previously described in Patel et al., Proc. Nat'l Acad. Sci. USA 1996, 93:2969-2974.

The 3' portion hybridizes to the template at a location 5' to the position or region in the template polynucleotide that is complementary to the 3' end of the primer extension product prior to switching template from the template polynucleotide to the unhybridized portion of the TSO ("termination site").

In one embodiment, strand switch is promoted by the presence of mutually complementary short sequences in the TSO segments immediately 5' and 3' to the junction between the hybridized and non-hybridized portions of the TSO. Without intending to be bound by theory, one explanation is that in the event that the primer extension product is extended into the portion of the target nucleic acid that is hybridized to the TSO (through displacement of the hybridized portion of the TSO), the 3' end of the primer extension

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product would comprise a short sequence that can bind to its complementary short sequence in the segment of the TSO immediately adjacent to the junction between the hybridized and non-hybridized portions of the TSO. This increases the efficiency of template switching by increasing the probability that the primer extension product would switch to the TSO tail portion as a template. The length of the short complementary sequences is preferably from about 3 to about 20 nucleotides, more preferably from about 5 to about 15 nucleotides, and most preferably from about 7 to about 10 nucleotides. In some embodiments, length is at least about any of 1, 3, 5, 7, 10 nucleotides, with an upper limit of about any of 10, 15, 20, 25 nucleotides. It is understood that the length can be greater or less, as appropriate under the reaction conditions of the methods of this invention.

In some embodiments, the 5' portion of the TSO comprises a sequence (hereinafter "propromoter sequence"), that is designed for formation of a double stranded promoter of an RNA polymerase. This embodiment of the TSO would function both as a termination sequence and to provide a promoter template. In this embodiment, the propromoter sequence of the TSO serves as a template for incorporation of a propromoter sequence (generally complementary to the propromoter sequence of the template TSO) into the primer extension product. Subsequent hybridization of a TSO comprising a propromoter sequence that is hybridizable to the propromoter sequence of the primer extension product results in formation of a double stranded promoter capable of effecting transcription by a suitable RNA polymerase. Promoter sequences that allow transcription of a template DNA are known in the art, as are methods of obtaining and/or making them. Preferably, the promoter sequence is selected to provide optimal transcriptional activity of the particular RNA polymerase used. Criteria for such selection, i.e., a particular promoter sequence particularly favored by a particular RNA polymerase, are also known in the art. For example, the sequences of the promoters for transcription by T7 DNA dependent RNA polymerase and SP6 are known in the art. The promoter sequence can be from a prokaryotic or eukaryotic source.

In one embodiment, the promoter sequence is adjacent to a sequence that is designed to provide for enhanced, or more optimal, transcription by the RNA polymerase used. In some embodiments, the sequence is not related (i.e., it does not substantially hybridize) to the target nucleic acid. More optimal transcription occurs when

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transcriptional activity of the polymerase from a promoter that is operatively linked to said sequence is greater than from a promoter that is not so linked. The sequence requirements for optimal transcription are generally known in the art as previously described for various DNA dependent RNA polymerases, such as in U.S. Pat. Nos. 5,766,849 and 5,654,142.

In a preferred embodiment, a segment of the 3' portion of the TSO (including the entire 3' portion that hybridizes to target) that hybridizes to the template DNA is attached to the template DNA such that displacement of the TSO by the polymerase that effects primer extension is substantially, or at least sufficiently, inhibited. Suitable methods for achieving such attachment includes techniques known in the art, such as using a cytosine analog that contains a G-clamp heterocycle modification (described in Flanagan et al., Proc. Natl. Acad. Sci. USA 1999, 96(7):3513-8); and locked nucleic acids (described, e.g., in Kumar et al., Bioorg. Med. Chem Lett. 1998, 8(16):2219-22; and Wahlestedt et al., Proc. Natl. Acad. Sci. USA 2000, 97(10):5633-8). Other suitable methods include using, where appropriate, sequences with a high GC content and/or cross-linking. Any of these methods for obtaining enhanced attachment may be used alone or in combination. Displacement of the TSO is substantially or sufficiently inhibited if the polymerase switches template from the target nucleic acid strand to the unhybridized portion of the TSO in at least about 25%, preferably at least about 50%, more preferably at least about 75%, and most preferably at least about 90%, of the events of primer extension. Substantially or sufficiently inhibited TSO displacement can also be empirically indicated if the amplification methods lead to a satisfactory result in terms of amount of the desired product. Generally, under a given set of conditions, the "modified" TSO binds more tightly to template as compared to a TSO not so modified.

The length of the TSO portion that hybridizes to the target nucleic acid strand is preferably from about 15 to 50 nucleotides, more preferably from about 20 to 45 nucleotides, and most preferably from about 25 to 40 nucleotides. In other embodiments, the length is at least about any of the following: 10, 15, 20, 25, 30; and less than about any of the following: 35, 40, 45, 50, 55. It is understood that the length can be greater or less, as appropriate under the reaction conditions of the methods of this invention. The complementarity of the TSO portion that hybridizes to the target nucleic acid strand is preferably at least about 25%, more preferably at least about 50%, even more preferably at

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least about 75%, and most preferably at least about 90%, to its intended binding sequence on the target nucleic acid.

Blocker sequence

In some embodiments, the primer extension termination sequence is provided by a blocker sequence. The blocker sequence is a polynucleotide, usually a synthetic polynucleotide, that is single stranded and comprises a sequence that is hybridizable, preferably complementary, to a segment of target nucleic acid sequence 5' of the position in the target sequence that is complementary to the 3' end of the primer extension product ("termination site"). The blocker comprises nucleotides that bind to the target nucleic acid with an affinity, preferably a high affinity, such that the blocker sequence resists displacement by DNA polymerase in the course of primer extension, in preferably more than about 30%, more preferably more than about 50%, even more preferably more than about 75%, and most preferably more than about 90%, of primer extension events. The length and composition of the blocker polynucleotide should be such that excessive random non-specific hybridization is avoided under the conditions of the methods of the present invention. The length of the blocker polynucleotide is preferably from about 3 to about 30 nucleotides, more preferably from about 5 to about 25 nucleotides, even more preferably from about 8 to about 20 nucleotides, and most preferably from about 10 to about 15 nucleotides. In other embodiments, the blocker polynucleotide is at least about any of the following: 3, 5, 8, 10, 15; and less than about any of the following: 20, 25, 30, 35. It is understood that the length can be greater or less as appropriate under the reaction conditions of the methods of this invention. The complementarity of the blocker polynucleotide is preferably at least about 25%, more preferably at least about 50%, even more preferably at least about 75%, and most preferably at least about 90%, to its intended binding sequence on the target nucleic acid.

In one embodiment, the blocker sequence comprises a segment that hybridizes to the target DNA is attached to the target DNA such that displacement of the blocker sequence by the polymerase that effects primer extension is substantially, or at least sufficiently, inhibited. Suitable means for achieving such attachment and determining

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substantial, or sufficient, inhibition of displacement are as described above for TSO used in the methods of the present invention.

In one embodiment, the blocker polynucleotide cannot function efficiently as a primer for nucleic acid extension (i.e., extension from the blocker sequence is reduced, or inhibited). Techniques for blocking the primer function of the blocker polynucleotide include any that prevent addition of nucleotides to the 3' end of the primer by a DNA polymerase. Such techniques are known in the art, including, for example, substitution or modification of the 3' hydroxyl group, or incorporation of a modified nucleotide, such as a dideoxynucleotide, in the 3'-most position of the blocker polynucleotide that is not capable of anchoring addition of nucleotides by a DNA polymerase.

Polynucleotide comprising a propromoter and a region which hybridizes to a displaced primer extension product

Some embodiments employ a polynucleotide comprising a propromoter and a region which hybridizes to a displaced primer extension product. In some embodiments, the polynucleotide is a TSO which contains a propromoter sequence, as discussed above. In other embodiments, the propromoter sequence is contained in a PTO, as described below.

Propromoter template oligonucleotide

In some embodiments, the methods employ a promoter sequence for transcription which is provided by a propromoter template oligonucleotide (PTO).

A PTO for use in the methods and compositions of the present invention is a single-stranded polynucleotide, generally DNA, comprising a propromoter sequence that is designed for formation of a ds promoter of an RNA polymerase, and a portion capable of hybridizing to the 3' end of the primer extension product. In a preferred embodiment, the propromoter sequence is located in the 5' portion of the oligonucleotide and the hybridizing sequence is located in the 3' portion of the oligonucleotide. In one embodiment, and most typically, the promoter and hybridizing sequences are different sequences. In another embodiment, the promoter and hybridizing sequences overlap in sequence identity. In yet another embodiment, the promoter and hybridizing sequences are the same sequence, and thus are in the same location on the PTO. In the embodiments wherein hybridization of the

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PTO to the primer extension product results in a duplex comprising an overhang (the 5' end of the PTO that does not hybridize to the displaced primer extension product, typically comprising all or part of the propromoter sequence), DNA polymerase fills in the overhang to create a double stranded promoter capable of effecting transcription by a suitable RNA polymerase.

Promoter sequences that allow transcription of a template DNA are known in the art and have been discussed above. Preferably, the promoter sequence is selected to provide optimal transcriptional activity of the particular RNA polymerase used. Criteria for such selection, i.e., a particular promoter sequence particularly favored by a particular RNA polymerase, is also known in the art. For example, the sequences of the promoters for transcription by T7 DNA dependent RNA polymerase and SP6 are known in the art. The promoter sequence can be from a prokaryotic or eukaryotic source.

In some embodiments, the PTO comprises an intervening sequence between a propromoter sequence and a portion capable of hybridizing to the 3' end of the primer extension product. Suitable length of the intervening sequence can be empirically determined, and can be at least about 1, 2, 4, 6, 8, 10, 12, 15 nucleotides. Suitable sequence identity of the intervening sequence can also be empirically determined, and the sequence is designed to preferably, but not necessarily, enhance degree of amplification as compared to omission of the sequence. In one embodiment, the intervening sequence is a sequence that is designed to provide for enhanced, or more optimal, transcription by the RNA polymerase used. Generally, the sequence is not related (i.e., it does not substantially hybridize) to the target nucleic acid. More optimal transcription occurs when transcriptional activity of the polymerase from a promoter that is operatively linked to said sequence is greater than from a promoter that is not so linked. The sequence requirements for optimal transcription are generally known in the art as previously described for various DNA dependent RNA polymerases, such as in U.S. Pat. Nos. 5766849 and 5654142, and can also be empirically determined.

In another embodiment, the PTO comprises a sequence that is 5' to the propromoter sequence, i.e., the PTO comprises additional nucleotides (which may or may not be transcriptional regulatory sequences) located 5' to the propromoter sequence. Generally, but not necessarily, the sequence is not hybridizable to the primer extension product.

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In one embodiment, the PTO cannot function efficiently as a primer for nucleic acid extension. Techniques for blocking the primer function of the PTO include any that prevent addition of nucleotides to the 3' end of the PTO by a DNA polymerase. Such techniques are known in the art, including, for example, substitution or modification of the 3' hydroxyl group, or incorporation of a modified nucleotide, such as a dideoxynucleotide, in the 3'-most position of the PTO that is not capable of anchoring addition of nucleotides by a DNA polymerase. It is also possible to block the 3' end using a label, or a small molecule which is a member of a specific binding pair, such as biotin.

The length of the portion of the PTO that hybridizes to the displaced primer extension product is preferably from about 5 to about 50 nucleotides, more preferably from about 10 to about 40 nucleotides, even more preferably from about 15 to about 35 nucleotides, and most preferably from about 20 to 30 nucleotides. In some embodiments, the hybridizing portion is at least about any of the following: 3, 5, 10, 15, 20; and less than about any of the following: 30, 40, 50, 60. The complementarity of the hybridizing portion is preferably at least about 25%, more preferably at least about 50%, even more preferably at least about 75%, and most preferably at least about 90%, to its intended binding sequence on the target nucleic acid.

DNA polymerase, ribonuclease and RNA polymerase

The amplification methods of the invention employs the following enzymes: a DNA polymerase, ribonuclease such as RNase H, and, optionally a DNA dependent RNA polymerase.

DNA polymerases for use in the methods and compositions of the present invention are capable of effecting extension of the composite primer according to the methods of the present invention. Accordingly, a preferred polymerase is one that is capable of extending a nucleic acid primer along a nucleic acid template that is comprised at least predominantly of deoxynucleotides. The polymerase should be able to displace a nucleic acid strand from the polynucleotide to which the displaced strand is bound, and, generally, the more strand displacement capability the polymerase exhibits (i.e., compared to other polymerases which do not have as much strand displacement capability) is preferable. Preferably, the DNA polymerase has high affinity for binding at the 3'-end of an oligonucleotide hybridized to a

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nucleic acid strand. Preferably, the DNA polymerase does not possess substantial nicking activity. Preferably, the polymerase has little or no 5'->3' exonuclease activity so as to minimize degradation of primer, termination or primer extension polynucleotides. Generally, this exonuclease activity is dependent on factors such as pH, salt concentration, whether the template is double stranded or single stranded, and so forth, all of which are familiar to one skilled in the art. Mutant DNA polymerases in which the 5'->3' exonuclease activity has been deleted, are known in the art and are suitable for the amplification methods described herein. Suitable DNA polymerases for use in the methods and compositions of the present invention include those disclosed in U.S. Pat. Nos. 5648211 and 5744312, which include exo$^-$ Vent (New England Biolabs), exo$^-$ Deep Vent (New England Biolabs), Bst (BioRad), exo$^-$ Pfu (Stratagene), Bca (Panvera), sequencing grade Taq (Promega), and thermostable DNA polymerases from thermoanaerobacter thermohydrosulfuricus. It is preferred that the DNA polymerase displaces primer extension products from the template nucleic acid in at least about 25%, more preferably at least about 50%, even more preferably at least about 75%, and most preferably at least about 90%, of the incidence of contact between the polymerase and the 5' end of the primer extension product. In some embodiments, the use of thermostable DNA polymerases with strand displacement activity is preferred. Such polymerases are known in the art, such as described in U.S. Pat. No. 5744312 (and references cited therein). Preferably, the DNA polymerase has little to no proofreading activity.

The ribonuclease for use in the methods and compositions of the present invention is capable of cleaving ribonucleotides in an RNA/DNA hybrid. Preferably, the ribonuclease cleaves ribonucleotides regardless of the identity and type of nucleotides adjacent to the ribonucleotide to be cleaved. It is preferred that the ribonuclease cleaves independent of sequence identity. Examples of suitable ribonucleases for the methods and compositions of the present invention are well known in the art, including ribonuclease H (RNase H).

The DNA-dependent RNA polymerase for use in the methods and compositions of the present invention are known in the art. Either eukaryotic or prokaryotic polymerases may be used. Examples include T7, T3 and SP6 RNA polymerases. Generally, the RNA polymerase selected is capable of transcribing from the promoter sequence provided by the

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TSO or PTO as described herein. Generally, the RNA polymerase is a DNA dependent polymerase, which is preferably capable of transcribing from a single stranded DNA template so long as the promoter region is double stranded.

In general, the enzymes used included in the methods and compositions of the present invention should not produce substantial degradation of the nucleic acid components of said methods and compositions.

Reaction conditions and detection

Appropriate reaction media and conditions for carrying out the methods of the present invention are those that permit nucleic acid amplification according to the methods of the present invention. Such media and conditions are known to persons of skill in the art, and are described in various publications, such as U.S. Pat. No. 5,679,512 and PCT Pub. No. WO99/42618. For example, a buffer may be Tris buffer, although other buffers can also be used as long as the buffer components are non-inhibitory to enzyme components of the methods of the invention. The pH is preferably from about 5 to about 11, more preferably from about 6 to about 10, even more preferably from about 7 to about 9, and most preferably from about 7.5 to about 8.5. The reaction medium can also include bivalent metal ions such as Mg^{2+} or Mn^{2+}, at a final concentration of free ions that is within the range of from about 0.01 to about 10 mM, and most preferably from about 1 to 5 mM. The reaction medium can also include other salts, such as KCl, that contribute to the total ionic strength of the medium. For example, the range of a salt such as KCl is preferably from about 0 to about 100 mM, more preferably from about 0 to about 75 mM, and most preferably from about 0 to about 50 mM. The reaction medium can further include additives that could affect performance of the amplification reactions, but that are not integral to the activity of the enzyme components of the methods. Such additives include proteins such as BSA, and non-ionic detergents such as NP40 or Triton. Reagents, such as DTT, that are capable of maintaining enzyme activities can also be included. Such reagents are known in the art. Where appropriate, an RNase inhibitor (such as Rnasine) that does not inhibit the activity of the RNase employed in the method can also be included. Any aspect of the methods of the present invention can occur at the same or varying temperatures. Preferably, the reactions are performed isothermally, which avoids the

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cumbersome thermocycling process. The amplification reaction is carried out at a temperature that permits hybridization of the oligonucleotides (primer, TSO, blocker sequence, and/or PTO) of the present invention to the template polynucleotide and that does not substantially inhibit the activity of the enzymes employed. The temperature can be in the range of preferably about 25°C to about 85°C, more preferably about 30°C to about 75°C, and most preferably about 37°C to about 70°C. In some embodiments that include RNA transcription, the temperature for the transcription steps is lower than the temperature(s) for the preceding steps. In these embodiments, the temperature of the transcription steps can be in the range of preferably about 25°C to about 85°C, more preferably about 30°C to about 75°C, and most preferably about 37°C to about 70°C.

Nucleotide and/or nucleotide analogs, such as deoxyribonucleoside triphosphates, that can be employed for synthesis of the primer extension products in the methods of the invention are provided in the amount of from preferably about 50 to about 2500 μM, more preferably about 100 to about 2000 μM, even more preferably about 500 to about 1700 μM, and most preferably about 800 to about 1500 μM. In some embodiments, a nucleotide or nucleotide analog whose presence in the primer extension strand enhances displacement of the strand (for example, by causing base pairing that is weaker than conventional AT, CG base pairing) is included. Such nucleotide or nucleotide analogs include deoxyinosine and other modified bases, all of which are known in the art. Nucleotides and/or analogs, such as ribonucleoside triphosphates, that can be employed for synthesis of the RNA transcripts in the methods of the invention are provided in the amount of from preferably about 0.25 to about 6 mM, more preferably about 0.5 to about 5 mM, even more preferably about 0.75 to about 4 mM, and most preferably about 1 to about 3 mM.

The oligonucleotide components of the amplification reactions of the invention are generally in excess of the number of target nucleic acid sequence to be amplified. They can be provided at about or at least about any of the following: 10, 10^2, 10^4, 10^6, 10^8, 10^{10}, 10^{12} times the amount of target nucleic acid. Composite primers, TSO, PTO and the blocker sequence can each be provided at about or at least about any of the following concentrations: 50 nM, 100 nM, 500 nM, 1000 nM, 2500 nM, 5000 nM.

In one embodiment, the foregoing components are added simultaneously at the initiation of the amplification process. In another embodiment, components are added in

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any order prior to or after appropriate timepoints during the amplification process, as required and/or permitted by the amplification reaction. Such timepoints, some of which are noted below, can be readily identified by a person of skill in the art. The enzymes used for nucleic acid amplification according to the methods of the present invention can be added to the reaction mixture either prior to the nucleic acid denaturation step, following the denaturation step, or following hybridization of the primer and/or blocker sequence to the target DNA, as determined by their thermal stability and/or other considerations known to the person of skill in the art.

The amplification reactions can be stopped at various timepoints, and resumed at a later time. Said timepoints can be readily identified by a person of skill in the art. Methods for stopping the reactions are known in the art, including, for example, cooling the reaction mixture to a temperature that inhibits enzyme activity. Methods for resuming the reactions are also known in the art, including, for example, raising the temperature of the reaction mixture to a temperature that permits enzyme activity. In some embodiments, one or more of the components of the reactions is replenished prior to, at, or following the resumption of the reactions. Alternatively, the reaction can be allowed to proceed (i.e., from start to finish) without interruption.

The detection of the amplification product is indicative of the presence of the target sequence. Quantitative analysis is also feasible. Direct and indirect detection methods (including quantitation) are well known in the art. For example, by comparing the amount of product amplified from a test sample containing an unknown amount of a polynucleotide containing a target sequence to the product of amplification of a reference sample that has a known quantity of a polynucleotide that contains the target sequence, the amount of target sequence in the test sample can be determined. The amplification methods of the present invention can also be extended to analysis of sequence alterations and sequencing of the target nucleic acid. Further, detection could be effected by, for example, examination of translation products from RNA amplification products.

Amplification methods of the present invention

The following are examples of the amplification methods of the invention. It is understood that various other embodiments may be practiced, given the general description

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provided above. For example, reference to using a composite primer means that any of the composite primers described herein may be used.

In one aspect of the present invention, a method for amplifying a nucleotide sequence complementary to a target nucleotide sequence is provided. In this method, isothermal linear nucleic acid sequence amplification is achieved. In another aspect, a method for amplifying a target polynucleotide sequence wherein the amplified product is sense RNA is provided, which is sometimes referred to herein as an "enhanced" linear amplification method. In one embodiment, a method of enhanced isothermal linear nucleic acid sequence amplification which is TSO-based is provided (hereinafter "Method 1"). In another embodiment, a method of enhanced isothermal linear nucleic acid sequence amplification which is blocker sequence and PTO-based is provided (hereinafter "Method 2").

Linear nucleic acid sequence amplification resulting in complementary DNA product

When not linked to transcription, the amplification method of the invention provides for isothermal linear amplification of a target nucleic acid sequence. The method utilizes a single composite primer. In one embodiment, the method also employs a termination sequence, such as a blocker sequence as described in Method 2, or a TSO, as described in Method 1. Methods 1 and 2 are described below. Insofar as the linear amplification is not linked to transcription, the components and steps leading to formation of a complex comprising a promoter sequence for a DNA dependent RNA polymerase, are not included.

The termination sequence (either TSO or blocker sequence component, if used) is added for producing a product of defined 3'-end. In some embodiments, natural sequence(s) within the template 5' of the primer binding site inhibits nucleic acid polymerization such that termination of primer extension is achieved. Such natural sequences are known in the art, for example, GC rich sequences, or can be empirically determined. Use of a termination sequence is particularly beneficial when amplifying genomic DNA so as to provide a defined end of primer extension. When this feature is not desired, the isothermal linear amplification according to the methods of the invention can

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be carried out without a termination sequence. The isothermal linear amplification further utilizes two enzymes, a DNA polymerase and a ribonuclease such as RNase H. Schematic description of the linear isothermal nucleic acid amplification of the invention is shown in Figure 1A-C.

Similar to Methods 1 and 2 as described below, the linear amplification method is designed to amplify a single stranded DNA target. When the target to be amplified is ds DNA, the target is first denatured to produce single stranded target. Target denaturation may be carried out using methods known in the art, such as heat or alkali treatment. When the target is single stranded RNA, such as mRNA or viral RNA, the target is first transcribed to produce cDNA target by methods known in the art.

As shown in Figure 1A-C, the linear isothermal amplification method of the invention comprises steps similar to the initial steps of the enhanced linear amplification methods (Methods 1 and 2) described below and in Figures 2A-C and 3A-D. The target nucleic acid is combined with a composite primer, DNA polymerase, a ribonuclease such as RNase H and optionally a blocker sequence component or TSO, as described above. In one embodiment, each amplification reaction includes composite primers of one identical sequence. In another embodiment, each amplification reaction includes a mixture of composite primer variants, wherein the variants represent two or more homologous but non-identical sequences, and wherein all are capable of hybridizing to the same target nucleic acid sequence. The complementarity is preferably at least about 50%, more preferably at least about 75%, and most preferably at least about 90%. Advantages of this embodiment include the ability to introduce different sequences of interest into the primer extension products. In yet another embodiment, each amplification reaction includes a mixture of composite primers, wherein the primers represent two or more non-identical sequences that are of low or no homology, and wherein the primers preferentially hybridize to different target nucleic acid sequences or different sites along the same target nucleic acid strand. Advantages of this embodiment include multiplex detection and/or analysis of target nucleic acids through amplification of a plurality of target nucleic acid species in a single amplification reaction.

Figure 1A-C illustrates an embodiment that includes a termination sequence. The composite primer and the termination sequence (TSO or blocker sequence component)

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hybridize to the same strand of the target, to form a tri molecular complex, XX (Figure 1A-C). The 3'-end of the composite primer is extended along the target strand by the polymerase, up to the site of hybridizing of the TSO or blocker sequence component, to yield complex XXI (Figure 1A-C). A ribonuclease such as RNase H cleaves the RNA, generally the 5'-RNA, portion of the extended primer of complex XXI (Figure 1A-C) to produce complex XXII (Figure 1A-C). A second composite primer binds to complex XXII (Figure 1A-C) by hybridization of the RNA, generally the 5' RNA, portion to yield complex XXIII (Figure 1A-C). The free 3' portion of the bound composite primer then displaces the 5' end of the primer extension product and hybridizes to the target to form complex XXIV (Figure 1A-C). The hybridization of the 3' end of the composite primer to the target is generally favored over the hybridization of the 5' end of the primer extension product since the hybridized 3' end of the primer is a site of binding of the DNA polymerase which will then extend the 3' end of the primer along the target. Primer extension results in displacement of the first primer extension product to yield complex XXV (Figure 1A-C). The process is repeated to yield multiple single stranded DNA displacement products which are generally complementary to the target sequence.

The single stranded DNA (i.e., the displaced primer extension products) of the isothermal linear amplification method are readily detectable by any of many detection methods known in the art. Various homogeneous or heterogeneous detection methods suitable for the detection of single stranded nucleic acid molecules were previously described, including identification by size and/or migration properties in gel electrophoresis, or by hybridization to sequence-specific probes.

The detection of the amplification product is indicative of the presence of the target sequence. Quantitative analysis is also feasible. For example, by comparing the amount of product amplified from a test sample containing an unknown amount of a polynucleotide containing a target sequence to the product of amplification of a reference sample that has a known quantity of a polynucleotide that contains the target sequence, the amount of target sequence in the test sample can be determined. The amplification methods of the present invention can also be extended to analysis of sequence alterations and sequencing of the target nucleic acid.

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The production of at least 1, at least 10, at least about 100, at least about 1000, at least about 10^5, at least about 10^7, at least about 10^9, at least about 10^{12}, complementary copies of each copy of template polynucleotide can be expected, thus leading to at least 1, at least 10, at least 100, at least 1000, at least about 10^5, at least about 10^7, at least about 10^9, at least about 10^{12}-fold enhancement with respect to each copy of template polynucleotide.

Enhanced linear amplification resulting in sense RNA product

The present invention also provides methods for amplifying a target polynucleotide sequence wherein the amplified product is RNA containing the sense sequence (i.e., same sequence as target). Amplification of target nucleic acid according to Method 1, which results in the generation of a unique intermediate amplification product comprising target and template switch oligonucleotide (TSO)-related portions, provides for coupling of the linear amplification to transcription. The complex formed by the hybridization of the template switch oligonucleotide and the displaced primer extension product is a substrate for transcription by the RNA polymerase, which generates an RNA product of the same sense as the initial target sequence. Similarly, amplification of nucleic acid target according to Method 2 results in formation of a displaced primer extension product which when hybridized to the promoter template oligonucleotide forms a complex, which is a substrate for the RNA polymerase. As in Method 1, this process results in coupling of the linear amplification to transcription. The production of preferably at least about 1, more preferably at least about 50, even more preferably at least about 75, still more preferably at least about 100, and most preferably at least about 1000, RNA transcript products from each primer extension product is expected, thus leading to preferably at least about 1, more preferably at least about 50, even more preferably at least about 75, still more preferably at least about 100, and most preferably at least about 1000-fold enhancement with respect to the non-transcription linked methods of amplification.

Below are two exemplary methods.

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Method 1-TSO-based enhanced linear nucleic acid amplification

In one embodiment, the TSO-based linear amplification method of the present invention is linked to transcription from the primer extension products to provide enhanced nucleic acid amplification. A schematic description of this novel amplification method, Method 1, is shown in Figure 2A-C.

The TSO-based nucleic acid amplification method of the invention employs a single composite primer, as described above. A second oligonucleotide used in the amplification method of the invention is a template switch oligonucleotide (TSO), also as described above. The amplification method of the invention employs the following enzymes: a DNA polymerase, a ribonuclease such as RNase H, and a DNA dependent RNA polymerase. The nucleic acid target to be amplified can be DNA or RNA. Amplification of an RNA target will require initial cDNA synthesis, as known in the art.

The new TSO-based enhanced linear amplification method of the present invention can produce multiple copies of an RNA product homologous (i.e., sense) to the target DNA sequence.

The single stranded target nucleic acid is combined with the composite primer, a TSO oligonucleotide, DNA polymerase, ribonuclease such as RNase H, a DNA dependent RNA polymerase, and nucleotides, such as deoxyribonucleoside triphosphates (dNTPs) and ribonuceoside triphosphates (rNTPs), in a reaction medium suitable for nucleic acid hybridization and amplification, as known in the art. Suitable reaction medium and conditions are as described above. In one embodiment, transcription is performed at a different temperature, generally lower, than that of the preceding steps. In another embodiment, all the steps of the methods are performed isothermally.

In one embodiment, each amplification reaction includes composite primers of one identical sequence. In another embodiment, each amplification reaction includes a mixture of composite primer variants, wherein the variants represent two or more homologous but non-identical sequences, and wherein all are capable of hybridizing to the same target nucleic acid sequence. The homology (sequence identity) is preferably at least about 50%, more preferably at least about 75%, and most preferably at least about 90%. Advantages of this embodiment include the ability to introduce different sequences of interest into the

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primer extension products. In yet another embodiment, each amplification reaction includes a mixture of composite primers, wherein the primers represent two or more non-identical sequences that are of low or no homology, and wherein the primers preferentially hybridize to different target nucleic acid sequences or different sites along the same target nucleic acid strand. Advantages of this embodiment include multiplex detection and/or analysis of target nucleic acids through amplification of a plurality of target nucleic acid species in a single amplification reaction.

In one embodiment, the TSO functions as a termination sequence and provides a propromoter sequence. In another embodiment, the TSO does not comprise a propromoter sequence. In this embodiment, a propromoter sequence is provided separately by another oligonucleotide, such as a PTO, that comprises a propromter sequence and is hybridizable to the 3' portion of the primer extension product such that transcription of the primer extension product can occur.

The single composite primer and the TSO then hybridize to the same strand of the nucleic acid to be amplified. The two oligonucleotides may be added to the sample suspected of containing the nucleic acid target prior to the target nucleic acid denaturation step. Hybridization of the two oligonucleotides to the target strand results in the formation of the tri molecular complex I (Figure 2A-C).

A DNA polymerase carries out primer extension. The primer is extended along the target nucleic acid strand of complex I (Figure 2A-C), up to the site of TSO hybridization. Template switching from the target strand to the 5' unhybridized portion of the TSO, and further primer extension along the TSO template results in the formation of the tri molecular complex II. The last comprises a target nucleic acid, the TSO and the first primer extension product. The first primer extension product is a unique DNA comprising both a target dependent portion (i.e., sequence complementary to the target nucleic acid) and a TSO dependent portion (i.e., sequence complementary to the unhybridized portion of the TSO) .

Complex II (Figure 2A-C) is a substrate for both an RNA polymerase and a ribonuclease such as RNase H. The DNA dependent RNA polymerase binds to the functional ds promoter of complex II and transcribes the first primer extension product to produce a sense RNA product III (Figure 2A-C). A ribonuclease, such as RNase H, which

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is specific for degradation of the RNA strand of an RNA/DNA heteroduplex, degrades the 5' portion of the primer extension product in complex II to form the tri molecular complex IV.

Free composite primer hybridizes to the primer complementary site of the target nucleic acid in complex IV (Figure 2A-C). This hybridization results in formation of complex V (Figure 2A-C) in which only the RNA portion, generally the 5' RNA portion, of the primer is hybridized to the target strand. Displacement of the 5' most portion of the primer extension product by the 3' DNA portion of the partially hybridized primer will result in formation of complex VI (Figure 2A-C), which is a substrate for a DNA polymerase. Extension of the primer along the target strand (VII; Figure 2A-C) results in displacement of the first primer extension product from the complex. Repeated primer extensions and strand displacements result in generation of multiple copies of polynucleotides that are at least substantially complementary to the target nucleic acid.

The primer extension product generated as described above is used as a template for transcription in the embodiment wherein TSO that comprises a propromoter sequence is provided. The displaced primer extension product (VIII; Figure 2A-C) hybridizes to free TSO oligonucleotide to form the partial duplex IX (Figure 2A-C). Complex (duplex) IX comprises a double stranded portion at one end and two non-complementary single strands respectively derived from the primer extension product and the TSO. The double stranded portion of this partial duplex contains a fully functional double stranded promoter for the DNA dependent RNA polymerase. The last binds to the promoter of the partial duplex IX and transcribes the primer extension product to form multiple copies of a sense RNA product X (Figure 2A-C).

The products of the amplification described above can be detected by either homogenous or heterogeneous detection methods, including identification by size and/or migration properties in gel electrophoresis, or by hybridization to sequence-specific probes. The detection of the amplification product is indicative of the presence of the target nucleic acid. Quantitative analysis is also feasible. For example, by comparing the amount of product amplified from a test sample containing an unknown amount of a polynucleotide containing a target sequence to the product of amplification of a reference sample that has a known quantity of a polynucleotide that contains the target sequence, the amount of target

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sequence in the test sample can be determined. Further extension of the new amplification method to analysis of sequence alteration and sequencing of the target nucleic acid are also feasible, as described below.

Method 2 –Blocker sequence-based enhanced nucleic acid amplification

In another embodiment, the blocker sequence-based linear amplification method of the present invention is linked to transcription from the primer extension products to provide enhanced nucleic acid amplification. This alternative enhanced linear amplification, Method 2, which does not involve a template switch step, is shown in Figure 3A-D.

Method 2 utilizes the single composite primer, as in Method 1, as described above, a blocker sequence component which is either an oligonucleotide or an oligonucleotide analog, which, as described above, is further able to hybridize to a sequence on the same target nucleic acid strand as the single primer, and a third oligonucleotide, the promoter template (PTO), which, as described above, comprises a 3'-portion which is able to hybridize (and is preferably complementary) to the 3'-end of the displaced extension product and a 5'-portion which includes at its 5' end a sequence of a promoter for a DNA dependent RNA polymerase. As in the TSO described above, the sequence immediately adjacent to the promoter sequence is designed to provide for preferably optimal transcriptional activity by the RNA polymerase used in the amplification according to the method of the invention. The blocker sequence component is designed to hybridize to the target sequence at a site which is located upstream, towards the 5' end of the target, relative to the site of hybridization of the single primer. Stated alternatively, and as described above, the blocker sequence hybridizes to a segment of target nucleic acid sequence 5' of the position in the target sequence that is complementary to the 3' end of the primer extension product. The blocker sequence binds with sufficiently high affinity so as to block primer extension at the site of blocker hybridization to the target. This feature provides a strong stop for primer extension by the polymerase and defines the 3'-end of the primer extension product.

As in Method 1, the target nucleic acid for amplification according to Method 2 is a single stranded DNA. When the target nucleic acid is a ds DNA, the target is first rendered

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single stranded by denaturation. The denaturation of the ds DNA target may be carried out by heating or any other known method known in the art, such as alkali treatment. When the target nucleic acid is RNA, such as mRNA, the target is first reverse transcribed by methods known in the art to produce a cDNA that is then amplified according to the method of the invention.

The single stranded nucleic acid target is combined with the single composite primer, the blocker component, the propromoter template (PTO), DNA polymerase, ribonuclease such as RNase H, a DNA dependent RNA polymerase, and nucleotides, such as NTPs (e.g., dNTPs and rNTPs), as was described for Method 1. Suitable reaction medium and conditions are as described above. In one embodiment, the transcription is performed at a different temperature, generally lower, than that of the preceding steps. In another embodiment, all the steps of the methods are performed isothermally.

In one embodiment, each amplification reaction includes composite primers of one identical sequence. In another embodiment, each amplification reaction includes a mixture of composite primer variants, wherein the variants represent two or more homologous but non-identical sequences, and wherein all are capable of hybridizing to the same target nucleic acid sequence. The homology (sequence identity) is preferably at least about 50%, more preferably at least about 75%, and most preferably at least about 90%. Advantages of this embodiment include the ability to introduce different sequences of interest into the primer extension products. In yet another embodiment, each amplification reaction includes a mixture of composite primers, wherein the primers represent two or more non-identical sequences that are of low or no homology, and wherein the primers preferentially hybridize to different target nucleic acid sequences or different sites along the same target nucleic acid strand. Advantages of this embodiment include multiplex detection and/or analysis of target nucleic acids through amplification of a plurality of target nucleic acid species in a single amplification reaction.

The single composite primer and the blocker sequence component hybridize to the same target strand to form a tri molecular complex. The primer is extended along the target up to the site of hybridization of the blocker sequence, to form complex XII (Figure 3A-D).

As in Method 1, a ribonuclease, such as RNase H, cleaves the RNA portion, generally the 5' RNA portion, of the single composite primer of complex XII to form

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complex XIII (Figure 3A-D). As described above, the enzyme is specific for cleaving the RNA strand of an RNA/DNA hybrid, and does not digest single stranded RNA. Thus, the ribonuclease does not degrade the free composite primer. The following steps, as illustrated in Figure 3A-D, of primer hybridization (XIV), displacement of the 5' end of the primer extension product by the 3'-DNA portion of the composite primer (XV), primer extension and displacement of the first primer extension product (XVI), proceed as in Method 1, to yield multiple copies of the displaced extension product (XVII). Unlike the displacement product of Method 1, XVII is fully complementary to the target sequence and does not comprise a 3' end portion which is not complementary to the target. Repeated primer extensions and strand displacements result in generation of multiple copies of polynucleotides that are complementary to the target nucleic acid.

The promoter template oligonucleotide (PTO) binds to the displaced extension product to form complex XVIII (Figure 3A-D), by hybridization of the 3' end portion (A) of the propromoter template to the 3' end of the displaced primer extension product. As described above, the 3' end of the PTO may be blocked or not. When the 3' end of the propromoter template is not blocked, the template will be extended along the displaced primer extension product. The 3' end of the displaced product will be extended by the nucleotide (DNA) polymerase along the B portion (see Figure 3A-D) of the hybridized propromoter template to form complex XIX, which comprises at its one end a ds promoter sequence that can be utilized by the DNA dependent RNA polymerase. Complex XIX is depicted in Figure 3A-D as the product of hybridization of a promoter template in which the 3' end is blocked for extension by the polymerase. Alternatively, when the 3' end of the promoter template is not blocked extension of the 3' end along the displaced primer extension product results in formation of a fully double stranded complex. DNA-dependent RNA polymerase will transcribe the extended displaced primer extension product of complex XIX, in both forms (the choice of RNA polymerase must take into account its capability to transcribe from a ds and/or ss DNA template), that is to say either the partial duplex or the fully double stranded duplex forms of the complex. Multiple copies of a single stranded RNA products are produced by this transcription step.

The products of the amplification described above can be detected by either homogenous or heterogeneous detection methods, including identification by size and/or

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migration properties in gel electrophoresis, or by hybridization to sequence-specific probes. The detection of the amplification product is indicative of the presence of the target nucleic acid. Quantitative analysis is also feasible. For example, by comparing the amount of product amplified from a test sample containing an unknown amount of a polynucleotide containing a target sequence to the product of amplification of a reference sample that has a known quantity of a polynucleotide that contains the target sequence, the amount of target sequence in the test sample can be determined. The amplification methods of the present invention can also be extended to analysis of sequence alterations and sequencing of the target nucleic acid, as discussed below.

Methods using the amplification methods and compositions of the invention

The methods and compositions of the present invention can be used for a variety of purposes. For purposes of illustration, methods of sequencing, genotyping (nucleic acid mutation detection), microarray preparation using the amplified nucleic acid products generated by the methods of the present invention, and characterizing nucleic acid sequences are described.

Isothermal sequencing of defined nucleic acid targets using the linear amplification method of the invention

The linear isothermal amplification methods of the invention are useful, for example, for sequencing of a defined nucleic acid target sequence. The sequencing process is carried out as described for the amplification methods described herein. In addition to the nucleotides, such as natural deoxyribonucleotide tri phosphates (dNTPs), that are used for the amplification method according to the present invention, the sequencing reaction mixture also includes the appropriate nucleotide tri phosphate analogs, which may be labelled or unlabelled, that upon incorporation into a primer extension product effect termination of nucleotide polymerization. Such analogs are commonly used in other sequencing methods and are well known in the art, such as dideoxyribonucleotides. They may be labeled, e.g., with fluorochromes or radioisotopes. The labels may also be labels which are suitable for mass spectroscopy. The label may also be a small molecule which is a member of a specific binding pair, and can be detected following binding of the other

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member of the specific binding pair, such as biotin and streptavidin, respectively, with the last member of the binding pair conjugated to an enzyme that catalyzes the generation of a detectable signal that could be detected by methods such as colorimetry, fluorometry or chemiluminescence. All of the above examples are well known in the art. These are incorporated into the primer extension product by the polymerase and serve to stop further extension along the target sequence. The resulting truncated extension products are labeled. The accumulated multiple displaced primer extension products vary in length, according to the site of incorporation of each of the analogs, which represent the various sequence locations of a complementary nucleotide on the target sequence.

Analysis of the reaction products for elucidation of sequence information can be carried out using any of various methods known in the art. Such methods include gel electrophoresis and detection of the labeled bands using appropriate scanner, sequencing gel electrophoresis and detection of the radiolabeled band directly by phosphorescence such as Molecular Dynamics reader, capillary electrophoresis adapted with a detector specific for the labels used in the reaction, and the like. The label can also be a ligand for a binding protein which is used for detection of the label in combination with an enzyme conjugated to the binding protein, such as biotin-labeled chain terminator and streptavidin conjugated to an enzyme. The label is detected by the enzymatic activity of the enzyme, which generates a detectable signal. As with other sequencing methods known in the art, the sequencing reactions for the 4 nucleotide types (A, C, G, T) are carried out either in a single reaction vessel, or in separate reaction vessels (each representing 1 of the 4 nucleotide types). The choice of method to be used is dependent on practical considerations readily apparent to one skilled in the art, such as the nucleotide tri phosphate analogs and/or label used. Thus, for example, when each of the analogs is differentially labeled, the sequencing reaction can be carried out in a single vessel. The considerations for choice of reagent and reaction conditions for optimal performance of sequencing analysis according to the methods of the invention are similar to those for other previously described sequencing methods. The reagent and reaction conditions should be as described above for the linear nucleic acid amplification methods of the present invention.

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Isothermal sequencing of defined nucleic acid targets using the enhanced linear amplification methods (methods 1 and 2) of the invention

Transcription based sequencing was previously described in, for example, Sasaki et. al., PNAS, 95:3455-3460, 1998. The inclusion of rNTPs analogs, which may be labelled or unlabelled, that upon incorporation into an RNA transcript effects termination of rNTP polymerization in the reaction mixture for the enhanced linear amplification methods will result in production of truncated RNA products, which result from blocking of the RNA polymerase at sites of incorporation of the analogs. Suitable rNTP analogs are known in the art. The last are incorporated opposite the complementary nucleotide on the displaced extension product in the relevant complexes according to the method used (Method 1 or Method 2).

Analysis of the reaction products for the elucidation of sequence information is carried out using any one of a variety of methods as known in the art. Such methods include those described above for sequencing of defined nucleic acid targets using the (non-enhanced) linear amplification methods of the invention.

RNA portion-based isothermal mutation detection utilizing the amplification methods of the invention

The unique properties of the composite primer for use in the isothermal amplification methods of the invention provide the basis for an isothermal method for the detection of defined mutations, or polymorphic sites (such as SNPs), in a target nucleic acid sequence. The method is useful for genotyping, detection of mutation leading to drug resistance and the like. These methods are applicable to characterizing sequences in a region in the template strand which generally hybridize to the RNA portion of the composite primer – hence reference to "defined" mutations, which are defined in terms of their location.

The target nucleic acid sequence suitable for the method of the invention is ss DNA. However, preparation of a ss DNA target from a ds DNA target or an RNA target can be carried out as described above and known in the art.

One embodiment of the method of the invention is schematically illustrated in Figure 4. In this embodiment, the RNA portion(s) of the composite primer is designed to

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be complementary to the sequence of the test target nucleic acid in which the presence of a sequence alteration is suspected. Stated alternatively, the primer comprises an RNA portion(s) that comprises a sequence that is complementary to the reference sequence (for example, a wild type sequence) against which the sequence in the test target nucleic acid is to be compared. In some embodiments, the altered sequence (i.e., the sequence comprising a sequence alteration) and the reference sequence are alleles. The sequence alteration may be a single nucleotide substitution, a deletion or insertion. The site of sequence alteration is schematically marked by X in Figure 4.

In another embodiment, the RNA portion(s) of the composite primer is designed to be complementary to the altered sequence suspected to be present in the test target nucleic acid. Stated alternatively, the primer comprises an RNA portion(s) that comprises a sequence that is complementary to the test target nucleic, and thus is not complementary to the reference sequence (for example, a wild type sequence) against which the sequence in the test target nucleic acid is to be compared. In some embodiments, the altered sequence (i.e., the sequence comprising a sequence alteration) and the reference sequence are alleles.

The RNA portion, generally 5' RNA portion, of the composite primer comprises a sequence which is complementary to a known normal wild type sequence, or a known mutant or a polymorphic genotype. Generally, a suitable composite primer comprises an RNA portion that allows the primer to preferentially hybridize to a target nucleic acid if the target nucleic sequence comprises a sequence complementary to the RNA portion of the primer compared to if there is a mismatch (i.e., the primer has the mutated sequence and the target does not, or vice versa), wherein the target nucleic acid has a bound primer extension product and has had its 5'-RNA portion cleaved. As shown in Figure 4, the presence of sequence alteration does not generally prevent the initial step of the amplification. The composite primer hybridizes to the target sequence, to form the first tri molecular complex, and is extended. A ribonuclease, such as RNase H, then cleaves the RNA portion of the extended primer of the complex. While it is likely that the presence of a mismatched base pair will affect the pattern of cleavage of the RNA/DNA hybrid, the cleavage is nonetheless likely to take place. The next step of binding of a composite primer to the complex by hybridization of the 5' RNA portion will be inhibited, preferably prevented, by a mismatch. This effect is dependent on factors such as the size of the

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hybridizing oligonucleotide and the stringency of the reaction condition. These factors are considered in the design of the composite primer, according to techniques well known and routine in the art. It is also possible that the mismatch will inhibit cleavage of the RNA portion(s) of the composite primer, thus preventing the amplification of the target sequence. Another possibility is that the mismatch will result in lower efficiency of cleavage of the RNA portion of the primer thus resulting in lower efficiency of amplification or production of less amplification product. The inability of the composite primer to hybridize to the target at this step of the amplification prevents further steps of primer extension strand displacement and production of multiple copies of the amplification products. It is understood that the detection of mutation by the methods of the present invention can be based on absence or presence of a primer extension product, or quantitative comparisons of amount of accumulated primer extension product. For example, when the composite primer comprises the reference sequence (for example, wild type), the presence of a mutation in a target strand may lead to no detectable amplification products; alternatively, it may lead to detectable products, but less than those produced from a template strand without the mutation.

When the composite primer comprises an RNA portion, generally a 5' RNA portion, that is fully complementary to a mutant genotype, amplification of a sequence which is of the normal genotype will be prevented, while a mutant genotype target will be amplified. Thus, in this case the detection and/or quantitative determination of multiple copies of the amplification product will be indicative of the presence of a target sequence of the mutant genotype. For example, parallel reactions that include either the nucleic acid sample of interest or reference sample of target nucleic with a wild type sequence could be run. Accumulation of more primer extension products in the former compared to the latter reaction would be indicative of the presence of a mutant genotype in the sample of interest. Alternatively, when the composite primer comprises a 5' RNA sequence that is fully complementary to a normal genotype sequence of the test target, amplification of a target sequence of the mutant genotype is prevented, and the detection and/or quantitative determination of amplification products is indicative of a normal genotype.

Any of the amplification methods of the present invention are suitable for detection of mutation as described above.

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3'-nucleotide-based isothermal mutation detection utilizing the amplification methods of the invention

In this method, the complementarity of the 3' most nucleotide of a composite primer is used to characterize the presence or absence of a mutated sequence. Hybridization of the 3' most nucleotide of the composite primer to a target nucleic acid is required for primer extension. Therefore, product amplification indicates that the target nucleic acid contains the sequence defined by the 3' most nucleotide of the composite primer. Reduction or absence of product amplification, on the other hand, indicates that the target nucleic acid contains a sequence alteration that includes at least the base complementary to the 3' most nucleotide of the composite primer. The lack of the sequence can be due to point mutation, single nucleotide polymorphism, insertion or deletion of a sequence segment encompassing the sequence defined by the 3' most nucleotide.

In one embodiment, genotype-specific primers, designed to have the 3' most nucleotide correspond to the various sequences at the variant nucleotide position (such as due to allelism), are used for amplification according to the methods of the invention. The presence of amplification products would indicate that the target nucleic acid comprises the sequence defined by the 3' most nucleotide of the particular primer used. The absence or lack (in comparison to a reference nucleic acid having the sequence defined by the 3' most nucleotide of the particular primer used) of amplification products would indicate that the target nucleic acid does not comprise the sequence defined by the 3' most nucleotide of the particular primer used.

Mutation detection based on single stranded conformation polymorphism utilizing the amplification methods of the invention

The DNA or RNA amplification products generated according to the methods of the present invention are also suitable for analysis for the detection of any alteration in the target nucleic acid sequence, as compared to a reference nucleic acid sequence which is identical to the target nucleic acid sequence other than the sequence alteration, as will be discussed in the following.

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The products of the linear nucleic acid amplification methods (DNA) and the enhanced linear amplification methods (RNA) described previously are suitable for single stranded conformation polymorphism (SSCP or rSSCP) based mutation detection. Insofar as the RNA product of the new amplification methods is not a substrate for further amplification, sequence amplification according to the new methods does not require the presence of agents which reduce secondary structures in the single stranded product. Transcription-based amplification methods described by others are carried out in the presence of agents which reduce secondary structures, such as DMSO. Thus, it is anticipated that the enhanced linear amplification methods of the present invention can be directly linked to appropriate means for detecting single stranded conformation polymorphism, such as an electrophoretic separation method for the identification of specific mobility pattern of the single stranded RNA products for the elucidation of the presence of specific sequence features, or the presence of any difference in a test nucleic acid as compared to a reference nucleic acid.

Methods based on gel electrophoresis or capillary electrophoresis can be used for the detection and analysis of the various single stranded conformational isomers. Alternatively, it is also likely that cleavage of the single stranded DNA or RNA product using nucleases which recognize sequence dependent secondary structures, may be useful for the determination of sequence specific conformation polymorphism. Such nucleases are known in the art, such as the Cleavase assay (Third Wave). The electrophoretic methods are potentially more suitable for high throughput mutation, or genotyping, detection methods.

The determination of sequence specific electrophoretic pattern for a given nucleic acid sequence is useful for the detection of specific alleles of a test sequence. Furthermore, it is expected that an electrophoretic mobility pattern for the various alleles could be well differentiated, thus allowing the detection of two alleles in a single genomic DNA sample, as required for heterozygous genotype, or multiple alleles. Any alteration in the test nucleic acid sequence, such as base substitution, insertions or deletion, could be detected using this method. The method is expected to be useful for detection of specific single base polymorphism, SNP, and the discovery of new SNPs.

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Method of preparing microarrays of nucleic acids

The single stranded nature of the products of the linear nucleic acid amplification methods (DNA) and the enhanced linear amplification methods (RNA) described previously are particularly suitable for preparing microarrays comprising the amplification products.

Several techniques are well-known in the art for attaching nucleic acids to a solid substrate such as a glass slide. One method is to incorporate modified bases or analogs that contain a moiety that is capable of attachment to a solid substrate, such as an amine group, a derivative of an amine group or another group with a positive charge, into the amplified nucleic acids. The amplified product is then contacted with a solid substrate, such as a glass slide, which is coated with an aldehyde or another reactive group which will form a covalent link with the reactive group that is on the amplified product and become covalently attached to the glass slide. Other methods, such as those using amino propryl silican surface chemistry are also known in the art, as disclosed at http://www.cmt.corning.com and http://cmgm.standord.ecu/pbrown1.

Attachment of groups to the primer which could be later converted to reactive groups is also possible using methods known in the art. Any attachment to nucleotides of the composite primer will become part of the single stranded DNA product of the linear amplifcation methods, which could then be attached to the solid surface of the microarray.

The amplification products of the methods of the present invention can be further modified, such as through cleavage into fragments or by attachment of detectable labels, prior to or following attachment to the solid substrate, as required and/or permitted by the techniques used.

Characterization of nucleic acids

The amplification products obtained by the methods of the invention are particularly amenable to further characterization, in part because the products are single stranded. The amplified products, either DNA or RNA, can analyzed using probe hybridization techniques known in the art, such as Southern and Northern blotting. The amplified products can also be analyzed by contacting them with microarrays comprising oligonucleotide probes. The identity of the probes provides characterization of the

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sequence identity of the amplified products, and thus by extrapolation the identity of the template nucleic acid present in a sample suspected of containing said template nucleic acid.

Compositions and kits of the invention

The invention also provides compositions and kits used in the methods described herein. The compositions may be any component(s), reaction mixture and/or intermediate described herein, as well as any combination. For example, the invention provides a composition comprising a composite primer, wherein the composite primer comprises an RNA portion and a 3' DNA portion. In another example, the invention provides a composition comprising a composite primer, wherein the composite primer comprises a 5'-RNA portion and a 3'-DNA portion. In one embodiment, the RNA portion is adjacent to the DNA portion. In another example, the invention provides a composition comprising a composite primer, wherein the composite primer comprises 5'- and 3'-DNA portions with at least one intervening RNA portion. In other examples, the invention provides a composition comprising a composite primer that is further derivatized by attachment of a moiety capable of effecting attachment of a polynucleotide comprising the composite primer to a solid substrate used in preparing nucleic acid microarrays. In some embodiments, the composite primer is further derivatized by attachment of a positively charged moiety such as an amine. In other embodiments, the invention provides a composition comprising a TSO (i.e., any of the TSO embodiments described herein, including TSOs containing one or more modifications which enhance binding to template). In some embodiments, the compositions comprise a composite primer and a termination sequence. In some embodiments, the invention provides a composition comprising a polynucleotide comprising a propromoter sequence, such as a TSO or PTO (i.e., any of those embodiments described herein), and may further comprise a composite primer and/or a blocker sequence. In some embodiments, the invention provides a composition comprising a blocker sequence (i.e., any of the embodiments described herein, including blocker sequences with modifications).

In other embodiments, the invention provides compositions comprising (a) a composite primer, wherein the composite primer comprises an RNA portion and a 3' DNA

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portion (in some embodiments, the RNA portion is adjacent to the DNA portion); and (b) a termination sequence. In some embodiments, the termination sequence is a TSO. In other embodiments, the termination sequence is a blocking sequence. In some embodiments, the composite primer comprises a 5'-RNA portion and a 3'-DNA portion (in certain embodiments, the RNA portion is adjacent to the DNA portion). In other embodiments, the composite primer comprises 5'- and 3'-DNA portions with at least one intervening RNA portion. In some embodiments, the composition comprises (a) a composite primer; (b) a polynucleotide comprising a termination sequence; (c) a polynucleotide comprising a propromoter sequence. In some embodiments, the propromoter sequence is provided by a PTO. In other embodiments, the propromoter sequence is provided by a TSO. Any of the above compositions may further comprise template (which comprises a target sequence) and/or any of the enzymes described herein (such as DNA polymerase, RNaseH, and/or RNA polymerase). The compositions are generally in aqueous form, preferably in a suitable buffer.

The invention also provides compositions comprising the amplification products described herein. Accordingly, the invention provides a population of DNA (anti-sense) or RNA (sense) molecules which are copies of a target sequence, which are produced by any of the methods described herein.

The compositions are generally in a suitable medium, although they can be in lyophilized form. Suitable media include, but are not limited to, aqueous media (such as pure water or buffers).

The invention provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any one or more of the uses described herein, and, accordingly, may contain instructions for any one or more of the following uses: amplifying a nucleotide sequence; sequencing of amplified polynucleotides; and detection of sequence mutation in amplified polynucleotides.

The kits of the invention comprise one or more containers comprising any combination of the components described herein, and the following are examples of such kits. A kit may comprise any of the composite primers described herein. In some embodiments, a kit comprises two or more composite primers, which may or may not be

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separately packaged. In other embodiments, a kit comprises a composite primer and a termination sequence (any of those described herein). A kit may comprise a composite primer, a polynucleotide comprising a termination sequence, and a polynucleotide comprising a propromoter sequence (which may be a PTO or TSO). The composite primer may be labelled or unlabeled. Kits may also optionally include any of one or more of the enzymes described herein, as well as deoxynucleoside triphosphates and/or ribonucleoside triphosphates. Kits may also include one or more suitable buffers (as described herein). Kits useful for nucleic acid sequencing may optionally include labeled or unlabelled nucleotide analogs that upon incorporation into a primer extension product effect termination of nucleotide polymerization. One or more 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 any of the methods described herein. Each component can be packaged in separate containers or some components can be combined in one container where cross-reactivity and shelf life permit.

The kits of the invention may optionally include a set of instructions, generally written instructions, although electronic storage media (*e.g.*, magnetic diskette or optical disk) containing instructions are also acceptable, relating to the use of components of the methods of the present invention for the intended nucleic acid amplification, and/or, as appropriate, for using the amplification products for purposes such as nucleic acid sequencing and detection of sequence mutation. The instructions included with the kit generally include information as to reagents (whether included or not in the kit) necessary for practicing the methods of the presentation invention, instructions on how to use the kit, and/or appropriate reaction conditions.

The component(s) of the kit may be packaged in any convenient, appropriate packaging. The components may be packaged separately, or in one or multiple combinations. Where kits are provided for practicing the enhanced linear amplifications methods of the present invention, the RNA polymerase (if included) is preferably provided separately from the components used in the steps prior to the transcription steps.

The relative amounts of the various components in the kits can be varied widely to provide for concentrations of the reagents that substantially optimize the reactions that need

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to occur to practice the methods disclosed herein and/or to further optimize the sensitivity of any assay.

The invention also provides systems for effecting the methods described herein. These systems comprise various combinations of the components discussed above. For example, in some embodiments, the invention provides a system suitable for producing target polynucleotide sequence (or amplifying target polynucleotide sequence) comprising (a) a composite primer (any of those described herein), (b) DNA polymerase; and (c) ribonuclease. In some embodiments, the system further comprises a polynucleotide comprising a termination sequence (any of those described herein). In some embodiments, the system further comprises a polynucleotide comprising a propromoter sequence (which may be a PTO or TSO) and a DNA-dependent RNA polymerase. Any of the systems embodiments may also comprise a template (target) sequence, as described herein.

The invention also provides reaction mixtures (or compositions comprising reaction mixtures) which contain various combinations of components described herein. In some embodiments, the invention provides reaction mixtures comprising (a) a polynucleotide template; (b) a composite primer comprising a 3' DNA portion and an RNA portion; and (c) DNA polymerase. As described herein, any of the composite primers may be in the reaction mixture (or a plurality of composite primers), including a composite primer comprises a 5' RNA portion which is adjacent to the 3' DNA portion. The reaction mixture could also further comprise an enzyme which cleaves RNA from an RNA/DNA hybrid, such as RNase H. A reaction mixture of the invention can also comprise any of the polynucleotides comprising termination sequences described herein. Another example of a reaction mixture is (a) a displaced primer extension product (and, as such, contains at its 5' end sequence complementary to the 3' DNA portion of the composite primer, but not sequences complementary to the RNA portion of the composite primer); (b) a polynucleotide comprising a propromoter sequence (for example, a PTO); and (c) RNA polymerase. Other reaction mixtures are described herein and are encompassed by the invention.

The invention also includes compositions comprising any of the complexes (which are intermediates in the methods described herein) described herein. The complexes are schematically depicted in Figures 1-4. As an example, one complex of the invention is a

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complex comprising: (a) a template strand; and (b) a composite primer, said composite primer comprising a 3' DNA portion and an RNA portion. The composite primer may have an RNA portion which is 5' and adjacent to the 3" DNA portion. The complex may further comprise a polynucleotide comprising a termination sequence (such as a TSO or blocker sequence). The complex may also comprise a polynucleotide comprising a propromoter, such as a PTO.

The following Examples are provided to illustrate, but not limit, the invention.

EXAMPLES

Example 1: General Methods

The general methods that are described in this example are also utilized in other examples provided herein.

Buffers

Buffers that were used throughout the examples are made with the following materials.

TE Buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA

TBE Buffer: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3

FX Buffer: 20 mM Tris-SO_4, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.1% NP-40

Isothermal linear amplification using a single chimera primer, DNA polymerase and Rnase H

Sequence amplification was performed in 15 ul reactions containing 20 mM Tris-HCl, pH 8.5, 6.0 mM $MgCl_2$, 1.0 mM dATP, 1.0 mM dCTP, 1.0 mM dTTP, 0.8 mM dGTP, 0.2 mM dITP (dNTP's from Amersham), 0-6% DMSO, 0-8% glycerol, 0-100 ug/ml acetylated BSA (Ambion, Austin, TX), 0.6 Units/ul recombinant ribonuclease inhibitor (rRNasin, Promega, Madison, WI), 0.5-5 uM composite primer IA005, and 100-200 nM promoter-template oligonucleotide (PTO) IA015C. Composite primer IA005 is a 20-mer primer with the sequence of: ACGGAUGCGGUCUCCAGTGT (SEQ ID NO:1).

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Promoter-template oligonucleotide (PTO) IA015C is a 55-mer oligonucleotide with the sequence of:
ggAATTCTAATACgACTCACTATAgggAgAgCggTACgCTgATCAAAgATCCgTg-biotin (SEQ ID NO:12).

Reactions were assembled with all components except the two enzymes. After heating to 70°C or 99°C for 10 sec. in a programmable thermal cycler (GeneAmp 9600, Perkin Elmer), the reaction mixtures were cooled down to 55°C, 60°C or 65°C, as described in the individual examples. Upon attaining the lower temperature, 0.05-0.24 Unit of RNase H (diluted from the 5 U/ul stock solution using a diluent/storage solution: 10 mM Tris-HCl, pH 8.5, 30% glycerol; Hybridase thermostable RNase H, Epicentre Technologies, Madison, WI) and 1.0-5.0 Units Bca DNA polymerase (2 U/ul; Panvera, Madison, WI) were added. The reactions were incubated at 55°C-65°C for 30 minutes At the end of the incubation, reactions were cooled to 4°C. Reactions can remain at 4°C until RNA transcription step is desired.

Enhanced linear amplification by RNA transcription of the linear amplification ssDNA product

RNA transcription was performed at 37°C for 3 hours in 10 ul reactions containing 2.5 ul of the linear amplification reaction mixtures above, and 40 mM Tris-HCl, pH 8.5, 70 mM KCl, 5.0 mM DTT, 12 mM $MgCl_2$, 110 ug/ml BSA, 3 mM each rNTP (ATP, UTP, CTP, GTP, Amersham), 7.5% DMSO, 1 Unit/ul rRNasin (Promega, Madison, WI), and 20 Units T7 RNA polymerase (Ambion, Austin, TX).

DNA Templates

A sequence from the J-gene region of *E. coli* K12 was chosen as a DNA template for several of the following Examples. Three DNA templates were used for these experiments: a synthetic DNA target (IA013), a primarily single-stranded DNA (351 bases) template produced by PCR amplification, and genomic DNA from the K12 strain of *E. coli* (preparation described in Example 4). Synthetic DNA target IA013 comprises:

Spacer18-Spacer18CGGTACGCTGATCAAAGATCCGTGCAACAAATGTCA

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TGGTCATGGTCGTGTTGAGCGCAGCAAAACGCTGTCCGTTAAAATCCCGGCAG GGGTGGACACTGGAGACCGCATCCGT (SEQ ID NO:18). Spacer18 refers to polyoxyethylene spacers. These were added to the oligo in order to retard its mobility with respect to the 100-bp ssDNA product. The sequence of the aforementioned primarily single-stranded DNA (351 bases) template produced by PCR amplification is: **CGGTACGCTGATCAAAGATCCGT**GCAACAAATGTCATGGTCATGGTCGTGTT GAGCGCAGCAAAACGCTGTCCGTTAAAATCCCGGCAGGGGTGG**ACACT*GGAG ACCGCATCCGT***CTTGCGGGCGAAGGTGAAGCGGGCGAGCATGGCGCACCGGCA GGCGATCTGTACGTTCAGGTTCAGGTTAAACAGCACCCGATTTTCGAGCGTGAA GGCAACAACCTGTATTGCGAAGTCCCGATCAACTTCGCTATGGCGGCGCTGGG TGGCGAAATCGAAGTACCGACCCTTGATGGTCGCGTCAAACTGAAAGTGCCTG GCGAAACCC**AGACCGGTAAGCTATTCCGTATGCG** (SEQ ID NO:17) wherein the PCR primers are bolded and underlined and the composite primers are bolded, with RNA portion in italics.

Preparation of ssDNA target from PCR amplification product

Single-stranded DNA template for isothermal linear amplification was prepared by PCR amplification of a 351-bp segment of the *E. coli* J gene using the primers IA006 and IA004. Primer IA006 is a 23-mer with the sequence of: CGGTACGCTGATCAAAgATCCGT (SEQ ID NO:16). Primer IA004 is a 26-mer with the sequence of: CGCATACGGAATAGCTTACCGGTCT (SEQ ID NO:15).

PCR was performed in 100 ul reactions containing 20 mM Tris-SO_4, pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.1% NP-40, 2.0 mM $MgCl_2$, 300 uM each dNTP (dATP, dTTP, dCTP, dGTP), 5 Units Taq DNA polymerase, 400 nM primer IA006, 400 nM 5'-phosphate-primer IA004, and 0.2 ul of a crude lysate of *E. coli* K12 strain. A modified "touchdown PCR" protocol was used with the following parameters: 95°C for 5 seconds, 68°C for 1 minute for 5 cycles; 94°C for 5 seconds, 60°C for 30 seconds, 72°C for 1 minute for 5 cycles; 94°C for 5 seconds, 55°C for 30 seconds, 72°C for 1 minute for 40 cycles; 72°C for 15 minutes and then held indefinitely at 4°C. Primer IA004 was synthesized with a 5'-phosphate to protect the sense-strand from digestion by lambda exonuclease (Strandase kit, Novagen, Madison, WI). The Strandase digestion was performed according to the

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manufacturer's recommendation. Briefly, PCR product prepared as described above, was precipitated from the reaction mixture by the addition of 1/10 volume 3M sodium acetate, pH 5.2 and 0.6 volumes isopropanol, cooling to -20°C for 1 hour, and centrifuged at maximum speed in a microcentrifuge for 30 minutes. The DNA pellet was washed once with 75% ethanol, then air-dried briefly before resuspension in 80 ul water. Concentration was estimated from O.D. at 260 nm, and 60 Units of lambda exonuclease (Strandase, Novagen) was added. Digestion was allowed to proceed at 37°C for 20 minutes, reactions were then heated to 75°C for 10 minutes, and cooled to 4°C. Incubations were performed in a programmable thermal cycler (GeneAmp 9600, Perkin Elmer). Remaining DNA was purified using QiaQuick Nucleotide Removal Columns (Qiagen, Valencia, CA) following the manufacturer's recommended procedure and using the buffers provided with the kit (Qiagen, Valencia CA). Briefly, 10 volumes of Buffer PN (Qiagen) were added to the sample. The entire volume was then applied to a Qiagen spin column and centrifuged (6000 rpm 1 minute in a microcentrifuge). The filtrate was discarded, and the column was washed twice with 500 ul of Buffer PE (Qiagen). The column was then dried thoroughly by centrifugation at maximum speed for 3 minutes. The DNA was eluted in 50 ul Buffer EB (10 mM Tris-HCl, pH 8.5)(Qiagen). The concentration was estimated to be about 2.5 X 10^{12} copies/ 5ul from OD at 260 nm. Gel analysis revealed that significant dsDNA (less than half the total) remained, but the error in concentration was less than 2-fold. The DNA was diluted to 10^{10} copies/ 5ul in TE Buffer. Serial dilutions were prepared from the 10^{10} copy stock solution as needed. Concentration based on O.D. measurement was confirmed by limiting dilution PCR analysis.

Gel Electrophoresis

Amplification products were electrophoretically separated on Novex pre-cast 4-20% polyacrylamide gradient gels (Invitrogen, Carlsbad, CA; part no. EC62255) in 1X TBE Buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3) in a Novex electrophoresis apparatus (EI9001-XCell II Mini Cell, Novex). Reaction mixtures (5 ul) from linear amplification or transcription (enhanced linear amplification) were mixed with 1 ul of 6X Gel Loading Solution (40% sucrose, 0.25% bromophenol blue, 0.25% xylene

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cyanole), and the entire sample was immediately loaded into each well. Gels were subjected to 250V for approximately 5 minutes, until all samples had entered the gel, and the voltage was lowered to 175V for 45 minutes. Gels were removed from between the plastic plates and stained in 0.5 ug/ml ethidium bromide in 1X TBE Buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3). A dsDNA molecular size marker (Hi-Lo DNA Marker, Bionexus, San Leandro, CA) was included in one lane of each gel run. This marker contains 16 fragments of the following sizes: 50, 100, 200, 300, 400, 500, 750, 1000, 1400, 1550, 2000, 3000, 4000, 6000, 8000, and 10000 bp. Typically, 50-2000 bp could be resolved on the gels used.

Hybridization

Oligonucleotide probes for hybridization examples(IA010 for ssDNA products; IA014 for ssRNA products) were 5'-end-labelled using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and γ-^{32}P-ATP (adenosine 5'-[γ-^{32}P] triphosphate, triethylammonium salt, Amersham, Piscataway, NJ; PB10218, >5000 Ci/mmol, 10 mCi/ml). Primer IA010 is a 21-mer with the sequence of: ATGTCATGGTCATGGTCGTGT (SEQ ID NO:13). Primer IA014 is a 31-mer with the sequence of: CTCAACACGACCATGACCATGACATTTGTTG (SEQ ID NO:14). Labelling reactions (50 ul total volume) contained 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 1 ug oligo (147 pmol for primer IA010; 101 pmol for primer IA014), 250 uCi γ-^{32}P-ATP, and 30 Units T4 polynucleotide kinase. Incubation was at 37°C for 30 minutes, followed by removal of unincorporated nucleotide using QIAquick Nucleotide Removal Kit (Qiagen, Valencia, CA). The decay rate (cpm) was determined in a Packard Minaxi Tri-Carb 4000 Series liquid scintillation counter by Cherenkov counting of 1 ul of the labelled oligo.

Hybridization was performed in 30 ul reactions. Product DNA (or RNA) (10 ul) was added to 20 ul of probe mix. Reactions contained 100 mM NaCl and 10^6 cpm of probe (correcting for decay using a half-life of 14.3 days). After heating to 65°C 15 seconds, hybridization was allowed to proceed at 42°C for 30 minutes, followed by cooling to 4°C. These steps were performed in a programmable thermal cycler with a heated cover (GeneAmp 9600, Perkin Elmer). The entire volume of hybridization reaction was

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electrophoresed in 10% polyacrylamide gels in 1X TBE Buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3) at 150V for 3 hours. Gels were removed from the glass plates, wrapped in plastic wrap, and exposed to autoradiography film (BioMax MR, Kodak) at -20°C overnight (~16 hours) with two intensifying screens.

Example 2: Isothermal linear amplification

Isothermal linear amplification using a single composite primer, DNA polymerase, RNase H, and TSO or blocker was performed. Reaction mixtures containing all reaction components, as described above, as well as reaction mixtures without one of the key reagents such as composite primer, RNase H, or Bca DNA polymerase (Panvera, Madison, WI) were spiked with 10^{10} copies of synthetic ssDNA target (IA010 - sequence listed in Example 1). A negative control reaction containing all the reagents and no target ssDNA, was also included. The isothermal linear amplification of target DNA sequence was carried out as described above. Target denaturation was carried out at 70°C and the isothermal amplification was carried out at 65°C.

The amplification products were resolved by gel electrophoresis (Figure 5) (First lane: molecular weight ladder; lanes #1-4: no DNA, no primer, no RNase H, no Bca DNA polymerase, respectively; lane 5: contains all components). No amplification products were detected in reaction mixtures without primer, RNase H or Bca DNA polymerase.

Probe IA010 hybridization and autoradiography to the ssDNA product of the linear amplification method, as shown in Figure 6 (Lanes 1-4: no DNA, no primer, no RNase H, no DNA polymerase, respectively; lane 5: contains all components), verified the identity of the amplification product. The linear amplification of the synthetic oligonucleotide target in this experiment was done using a non blocked promoter-template oligonucleotide (IA015b). Promoter-template oligonucleotide IA015b is a 55-mer with a sequence of: GGAATTCTAATACGACTCACTATAGGGAGAGCGGTACGCTGATCAAAGATCCG TG (SEQ ID NO:11). The amplification reaction standard reaction components used for this amplification reaction are as given above. The initial denaturation step was performed at 70°C for 10 seconds. The reactions were cooled down to 65°C, and further incubated at this temperature for 30 minutes following the addition of Bca polymerase and RNase H. No

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hybridization was detected in the control reactions (no DNA, no primer, no RNase H, no Bca).

Example 3: Promoter-template oligonucleotide coupled isothermal linear amplification and transcription

The promoter-template oligonucleotide (PTO) contains two essential sequence motifs: a T7 promoter sequence (5'-TAATACGACTCACTATAGGGAgGAG (SEQ ID NO:20)) and a sequence complementary to the ssDNA template. Four versions of a PTO were designed (IA012, IA012b, IA015, IA015b). IA012 PTO is a 67-mer and has a sequence of: GGAATTCTAATACGACTCACTATAGGGAGAGATCGAGTAGCTCCGGTACGCTG ATCAAAGATCCGTG (SEQ ID NO:8). IA012 PTO contains two sequences in addition to the core T7 promoter: a 5'-extension (5'-GGAATTC (SEQ ID NO:21)) and a spacer (5'-ATCGAGTAGCTC (SEQ ID NO:22)) between the promoter and the target DNA-complementary sequence. IA015 is the shorter PTO (48-mer), lacking both the 5'-extension and the spacer. IA015 PTO has the sequence of: TAATACGACTCACTATAGGGAGAGCGGTACGCTGATCAAAGATCCGTG (SEQ ID NO:10). IA012b PTO is a 60-mer which contains the spacer, but not the extension. IA012b PTO has the sequence: TAATACGACTCACTATAGGGAGAGATCGAGTAGCTCCGGTACGCTGATCAAAG ATCCGTG (SEQ ID NO:9). IA015b contains the extension, but not the spacer. The sequence of IA015b is disclosed in Example 2. All primers other than the chimeric oligonucleotides IA005, IA019, and IA020 were synthesized by Keystone (Division of BioSource International, Camarillo, CA) and were PAGE purified.

A general schematic for this amplification method is illustrated in Figure 2A-C. The ability of IA012, IA012b, IA015, and IA015b to convert the ssDNA template into a substrate for T7 RNA polymerase was assessed by comparing the amount of RNA produced after transcription of overlap-extension products formed between a synthetic oligo product (IA009) and each of the PTO's. Synthetic oligo product IA009 is a 100-mer with the sequence of: AGTGTCCACCCCTGCCGGGATTTTAACGGACAGCG

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TTTTGCTGCGCTCAACACGACCATGACCATGACATTTGTTGCACGGATCTTTGA TCAGCGTACCG (SEQ ID NO:19). Overlap-extension was performed in 15 ul reactions containing 20 mM Tris-HCl, pH 8.5, 6 mM $MgCl_2$, 1 mM each dNTP (dATP, dTTP, dCTP, dGTP), 100 nM IA009, 100 nM PTO, and 1 Unit Bca DNA polymerase. Reactions were constituted without Bca DNA polymerase, heated to 95°C then cooled over 10 minutes to 60°C. After addition of DNA polymerase, reactions were incubated at 60°C for 30 minutes. A portion (2.5 ul) of the reaction mixture was added to the standard RNA transcription reaction mixture and the transcription reactions were assessed by gel electrophoresis.

As shown in Figure 7 (Lane 1: molecular weight ladder; lanes 2-5: overlap extension product from IA012, IA012b, IA015, IA015b, respectively; lanes 6-13: RNA from IA012, IA012b, IA015, IA015b, IA012, IA012b, IA015, IA015b, respectively) significantly more RNA was produced by the transcription substrate produced with the shorter PTO's (IA015, IA015b) than by either of IA012 or IA012b. The PTO containing the 5'-extension but not the spacer (IA015b) produced demonstrably higher yields of RNA. In all cases, however, multiple products appeared in addition to the major RNA band. A fifth PTO was designed having the same sequence as IA015b, but with a 3'-blocking group (biotin) to eliminated the free 3'-OH, to demonstrate the improved performance of a 3'-blocked PTO. Blocking the free 3'-OH of the PTO eliminates its ability to initiate non-specific incorporation of a functional promoter sequence to the amplifiction products leading to non-specific generation of transcription products. The performance of 3-blocked and unblocked PTO in the enhanced isothermal linear amplification was assessed from amplification of a synthetic oligonucleotide target, using the standard conditions. The 3'-blocked PTO (IA015c) produced comparable yields of specific RNA as IA015b, but with significantly less background, as shown in Figure 8 (Lane 1: molecular weight ladder; lanes 2, 9: no DNA, 30 minutes; lanes 3, 10: no DNA, 1 h; lanes 4, 11: no DNA, 2h; lanes 5, 12: 10^{10} copies IA013, 30 min; lanes 6, 13: 10^{10} copies IA013, 1h; lanes 7, 14: 10^{10} copies IA013, 2h; lanes 8, 15: 10^{11} copies IA013, 30 min; reactions 1-7 were not blocked (IA015b), reactions 8-15 were blocked (IA015c)). Negative control reactions (no DNA template) and reactions containing 10^{10} copies of oligo target (IA013) were amplified by strand displacement for 30 minutes, 1 hour, or 2 hours at 55C, with either IA015b or IA015c included in the strand-displacement reaction. When the 3'-OH was not blocked,

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non-specific RNA was produced in all reactions and obscured identification of the specific RNA band. In contrast, the blocked PTO produced primarily a single RNA product.

Example 4: Amplification of a J gene sequence of *E. coli* genomic DNA by the isothermal enhanced linear amplification

DNA was isolated from 25 ml of *E. coli* K12 (ATCC 10798) grown overnight in Tryptone-NaCl medium. Genomic DNA was isolated by lysozyme digestion and solubilization in a chaotropic lysis solution (Bactozol Kit, Molecular Research Center, Cincinnati, OH) following the manufacturer's recommended procedure. Briefly, bacteria were collected by centrifugation at 6000 x g for 5 minutes. Cell pellets were resuspended in Bactozyme digestion buffer and incubated at 50°C for 30 minutes. The resulting lysate was clear at the end of the digestion, without any visible clumps of undigested cells. The lysate was mixed with 4 volumes of DNazol reagent (Molecular Research Center, Cincinnati, OH) and incubated for 15 minutes at room temperature. DNA was precipitated from the solution by addition of 0.6 volume ice-cold ethanol. After incubation for 5 minutes at room temperature, the precipitated DNA was collected by centrifugation for 5 minutes at maximum speed in a micro-centrifuge. The DNA pellet was washed with 75% ethanol, centrifuged again, and resuspended in 1.5 ml TE Buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) by heating at 50°-55°C for 30 minutes, with frequent agitation. The resulting solution was passed repeatedly through a 22 gauge syringe needle to shear the DNA and reduce the viscosity of the solution. DNA was precipitated again (EPI005-35) by the addition of 1/10 volume of 5M ammonium acetate and 3 volumes ice-cold ethanol. After incubation at -20°C for 1 hr, the DNA was collected by centrifugation at maximum speed in a micro-centrifuge. The pellet was washed with 75% ethanol, centrifuged again, and resuspended in 150 ul TE Buffer. Two dilutions in TE Buffer were prepared for O.D. measurement (Beckman DU640 spectrophotometer), from which DNA concentration was calculated by assuming 50 ug/ml dsDNA produces an O.D. at 260 nm of 1. DNA concentrations of the two dilutions were 24.2 ug/10 ul and 24.6 ug/10 ul. The average of these two measurements (24.4 ug/ 10 ul) corresponds to approximately 2.5 X 10^9 genome copies / 5 ul (5 fg of *E. coli* genomic DNA = 1 copy).

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Isothermal enhanced linear amplification

DNA was serially diluted in TE Buffer to 10^9, 10^8, or 10^7 copies/ 5 ul, and denatured by heating to 95°C for 5 minutes followed by rapid cooling on ice. Single-stranded template DNA also was diluted to 10^9 copies/ 5ul. Reactions were assembled to contain no DNA, 10^7, 10^8, 10^9, or 2.5 X 10^9 copies of genomic DNA.

Amplification was performed in 15 ul reactions containing 20 mM Tris-HCl, pH 8.5, 6.0 mM $MgCl_2$, 1.0 mM dATP, 1.0 mM dCTP, 1.0 mM dTTP, 0.8 mM dGTP, 0.2 mM dITP (dNTP's from Amersham), 6% DMSO, 8% glycerol, 100 ug/ml acetylated BSA (Ambion, Austin, TX), 0.6 Units/ul recombinant ribonuclease inhibitor (rRNasin, Promega, Madison, WI), 5 uM composite primer IA005 (sequence disclosed in Example 1), 200 nM promoter-template oligonucleotide (PTO) IA015C (sequence disclosed in Example 1). Reactions were assembled with all components except the two enzymes. After heating to 99°C for 10 seconds in a programmable thermal cycler (GeneAmp 9600, Perkin Elmer), reactions were incubated at 60°C for 30 minutes Upon attaining 60°C, 0.6 ul of RNase H (0.05 Units diluted from the 5 U/ul stock solution in 10 mM Tris-HCl, pH 8.5, 30% glycerol), Hybridase, Epicentre Technologies, Madison, WI) and 1.0 ul Bca DNA polymerase (2.0 Units, Panvera, Madison, WI) were added. At the end of the 60°C incubation, reactions were cooled to 4°C. A volume of 5.0 ul of strand-displacement product was added to each RNA transcription reaction (total volume 20 ul). RNA transcription was performed using the standard conditions and scaling up the reaction volume to 20 ul to provide sufficient material for direct gel analysis (5 ul) and probe hybridization (10 ul).

Unlike the amplification of defined single stranded synthetic target, the amplification of genomic DNA according to the method of the invention requires the formation of a defined stop for the formation of a ssDNA product with a defined 3'-end. The formation of a defined stop for primer extension can be achieved by a blocker, which hybridizes to defined site on the target strand and can not be displaced by the polymerase. Alternatively, as in the present example, a GC rich sequence up stream of the primer site, provided a stop point for primer extension, thus leading to the formation of a ssDNA product with defined 3-end.

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Successful amplification of a defined sequence of genomic DNA by the enhanced isothermal linear amplification method of the invention is shown in Figure 9 (Lane 1: no DNA; lane 2: 10^7 copies E. coli genomic DNA; lane 3: empty; lane 4: 10^8 copies E. coli genomic DNA). The ssRNA product is shown to hybridize to a specific oligonucleotide probe.

Example 5: Evaluation of the effect of primer design on the performance of enhanced isothermal linear amplification

The performance of each of the three composite primers in the amplification methods of the invention was assessed. The isothermal linear amplification was performed in 15 ul reactions containing 20 mM Tris-HCl, pH 8.5, 6.0 mM $MgCl_2$, 1.0 mM dATP, 1.0 mM dCTP, 1.0 mM dTTP, 0.8 mM dGTP, 0.2 mM dITP (dNTP's from Amersham), 6% DMSO, 8% glycerol, 100 ug/ml acetylated BSA (Ambion, Austin, TX), 0.6 Units/ul recombinant ribonuclease inhibitor (rRNasin, Promega, Madison, WI), 5 uM composite primer, 200 nM promoter-template oligonucleotide (PTO) IA015C. The sequence of PTO IA015C is disclosed in Example 1. The sequence of composite primers IA005 (20-mer) is disclosed in Example 1. Other composite primer sequences with alphanumerical names are as follows:

IA019 (20-mer) ACGGAUGCGGUCUCCAGTGT (SEQ ID NO:2)

IA020 (21-mer) GACGGAUGCGGUCUCCAGTGT (SEQ ID NO:3)

Four other composite primer sequence were used that did not have alphanumerical names. Their sequences are, respectively:

(1) GCAAGACGGAUGCGGUCUCCAGTGT (SEQ ID NO:4)

(2) GACGATGCGUCTCCAGTGT (SEQ ID NO:5)

(3) GACGGATGCGGUCTCCAGUGT (SEQ ID NO:6)

(4) GACGGATGCGGUCTCCAGUGUCCA (SEQ ID NO:7)

These composite primers were synthesized by Dharmacon Research, Inc. (Boulder, CO). The RNA portion of the oligonucleotide was synthesized using a 5'-silyl protecting group in conjunction with an acid-labile 2'-orthoester protecting group (2'-bis(acetoxyethoxy)-methyl ether or "2'-ACE" (Scaringe, S.A., et al. *J. Am. Chem. Soc.* 120:11820-11821 (1998) and Scaringe, S.A. Advanced 5'-silyl-2'-orthoester approach to

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RNA oligonucleotide synthesis. Methods in Enzymology (in press)). Primers were PAGE purified.

Reactions were assembled with all components except the two enzymes. After heating to 70°C for 10 seconds in a programmable thermal cycler (GeneAmp 9600, Perkin Elmer), reactions were cooled to 55°C-65°C. Upon attaining the lower temperature, 0.05 Unit of RNase H (diluted from the 5 U/ul stock solution using a diluent/storage solution: 10 mM Tris-HCl, pH 8.5, 30% glycerol; Hybridase thermostable RNase H, Epicentre Technologies, Madison, WI) and 2.0 Units Bca DNA polymerase (2 U/ul; Panvera, Madison, WI) were added. The reactions were incubated at 55°C-65°C for 30 minutes. At the end of the incubation, reactions were cooled to 4°C until RNA transcription. RNA transcription was performed at 37°C for 3 hours in 10 ul reactions containing 2.5 ul of linear amplification reaction above, and 40 mM Tris-HCl, pH 8.5, 70 mM KCl, 5.0 mM DTT, 12 mM $MgCl_2$, 110 ug/ml BSA, 3 mM each rNTP (ATP, UTP, CTP, GTP, Amersham), 7.5% DMSO, 1 Unit/ul rRNasin (Promega, Madison, WI), and 20 Units T7 RNA polymerase (Novagen, Madison, WI).

The products of the enhanced linear amplification generated with each of the composite primers were resolved by gel electrophoresis as shown in Figure 10 (Lane 1: molecular weight ladder; lanes 1,2: IA015, 60°C; lanes 3,4: IA019, 55°C; lanes 5,6: IA019, 60°C; lanes 7,8: IA019, 65°C; lanes 9,10: IA020, 55°C; lanes 11, 12: IA020, 60°C; lanes 13,14: IA020, 65°C). The composite primers were designed to hybridize at the same site on the target strand, and differed by the number of deoxynucleotides at the 3'-end. The highest yield of RNA product was produced with primer IA020, followed by IA005 and IA019 equally. The other four composite primers yielded less RNA products. The optimal temperature for the isothermal linear amplification step was different for the different primers, as expected.

Example 6: Isothermal sequencing of nucleic acid targets using the linear amplification methods

Nucleic acid sequences to be analyzed are first isolated from their sources. Non-limiting examples of sources used in this example are animal, plant, bacteria, viruses, yeast, or fungi. If the source of nucleic acids (DNA or RNA) is from animal tissues, then the

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tissue is homogenized or sonicated or subjected to some force that enables single cells to become separated from the connective tissue. Caution is exercised to prevent degradation of DNA and RNA, especially mRNA, and, to prevent shearing of genomic DNA during the handling of the tissue. Animal cells can also be obtained from commercial sources, i.e. Gibco BRL, or from non-profit sources, i.e. American Tissue Type Culture (ATCC). Depending on the form of nucleic acid desired, genomic DNA, total RNA, or mRNA can be isolated by following standard protocols found in Sambrook et al. *supra*. Protocols for isolating nucleic acid from plant cells can also be found in Current Protocols in Molecular Biology *supra*. If the source of nucleic acid is from non-mammalian sources, for example, bacteria, yeast, fungi, or viruses, slightly different protocols are used to isolate nucleic acid. These protocols can be found in Sambrook et al. or Current Protocols in Molecular Biology for bacteria, yeast, fungi, and viruses.

Amplification of the defined target nucleic acid sequence is carried out as described for isothermal linear amplification, as described herein. About 10^2 to 10^{12} copies is used for template. In addition to the natural deoxyribonucleotide triphosphates (dNTPs) that are used for the amplification method, the sequencing reaction mixture includes the labeled triphosphate analogs. If each analog is uniquely labeled, all four can be added in the same reaction tube. Otherwise, if each nucleotide analog is labeled with the same label, the sequencing reactions are carried in four different reaction tubes in which each reaction mixture contains one of the nucleotide analogs. These analogs are incorporated to the primer extension product by the polymerase and serve to stop further extension along the target sequence. The resulting truncated extension products are labeled. The accumulated multiple displaced primer extension products vary in length, according to the site of incorporation of each of the analogs, which represent the various sequence locations of a complementary nucleotide on the target sequence. Analysis of the reaction products for elucidation of sequence information can be carried out by running the products on a gel. Alternatively, other methods of anlysis can be used as well. As with other sequencing methods, the sequencing reactions are carried out either in a single reaction vessel, or in separate reaction vessels. The choice of method to be used is dependent on the nucleotide triphosphate analogs used. Thus when each of the analogs is differentially labeled, the sequencing reaction can be carried out in a single vessel. The considerations for choice of

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reagent and reaction conditions for optimal performance of sequencing analysis according to the method of the invention are similar to those for other previously described methods.

The plurality of primer extension products which differ in size in accordance with the specific incorporation of elongation terminator, are size separated using any of a variety of methods known in the art. The profile of the plurality of primer extension products produced with each of the terminator analog is indicative of the nucleotide sequence of the test nucleic acid sequence.

Example 7: Sequencing of nucleic acid targets using enhanced amplification

Amplification of the defined target nucleic acid sequence is carried out as described for isothermal linear amplification, which involves transcription, as described herein. Either use of TSO's or PTO's may be used to append the protopromoter sequence to the product of the isothermal linear amplification. In addition to the natural ribonucleotide triphosphates (rRTPs) that are used for the enhanced linear amplification method according to the present invention, the sequencing reaction mixture also includes the appropriate labeled triphosphate analogs, which are commonly used in other sequencing methods known in the art. These are incorporated to the extension product by the RNA polymerase and serve to stop further extension along the target sequence. The resulting truncated extension products are labeled. The accumulated multiple displaced extension products vary in length, according to the site of incorporation of each of the analogs, which represent the various sequence locations of a complementary nucleotide on the target sequence. Analysis of the reaction products for elucidation of sequence information can be carried out as stated in the above sequencing example.

Example 8: Genotyping using isothermal enhanced linear amplification and genotype specific composite primer

Genomic DNA is isolated from test cells using methods described in previous examples or by other mean known to the skilled artisan. Different organisms including, but not limited to, bacteria, viruses, fungi, yeast, plants, and animals are genotyped. Genotype specific primers are designed either to comprise a 3'-end nucleotide which hybridizes to one genotype of a specific nucleic acid sequence, or hybridize to the counterpart genotype.

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The sequence variation determining specific genotypes may be point mutation, single nucleotide polymorphism (SNP), insertions deletions and the like.

Amplification of the defined target nucleic acid sequence is carried out as described for amplification of genomic E. coli sequence, in the above example. Using the genotype specific primer and DNA polymerase which is devoid of proof reading activity, the generation of amplification product indicates the presence of target sequence of the defined genotype. Sequence variation that prevents hybridization of the 3'-end of the primer to the target nucleic acid sequence will prevent amplification. The amplification product is detected by any one of various methods for detection of single stranded nucleic acid sequence, which is known in the art. For example, the hybridization of specific labeled oligonucleotide probes to the amplification product is detected by gel electrophoresis.

In cases where the genotyping of diploid cells is required, such as the determination of homozygote or heterozygote genotype, it is feasible to carry out the amplification of the specific nucleic acid target sequence using specific primers which are designed for either the wild type and mutant genotype, or one genotype and the other genotype. The amplification reactions using the specific primers are carried out in separate reaction vessels. The detection of amplification product in only one reaction tube or less amplification product is indicative of a homozygote genotype, i.e. either wild type or mutant homozygote. The detection of amplification product in both amplification reactions indicates a heterozygote genotype.

Example 9: RNA portion-based isothermal mutation detection utilizing amplification methods

The isothermal amplification methods disclosed herein are used for the detection of defined mutations, or polymorphic sites (such as SNPs), in a target nucleic acid sequence. The methods are used for genotyping or detection of mutation leading to drug resistance. The target nucleic acid sequence is single-stranded (ss) DNA. Single-stranded DNA target is prepared from a double-stranded DNA target or an RNA target using isothermal amplification methods described herein.

As shown in Figure 4, a composite primer which is complementary to a wild type DNA sequence is used in combination with a ss DNA target that has or is suspected to have

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a mutation within the allele under scrutiny. About 10^2 to about 10^{12} copies of the ssDNA target and about 0.05- 5 μM of composite primer is used. The presence of sequence alteration does not prevent the initial step of the amplification whereby the composite primer hybridizes to the target sequence to form the first tri-molecular complex, and an extension product is made. A ribonuclease, RNase H, then cleaves the RNA portion of the extended primer of the complex. The cleavage of the RNA portion of the composite primer, or the primer extension product, by RNase H is affected by the presence of a mismatch. This may prevent cleavage, alter cleavage patern, or reduce the efficiency of the cleavage. The next step of binding of a composite primer to the complex by hybridization of the 5' RNA portion is inhibited by a mismatch for the reason described above. The inability of the composite primer to hybridize to the target prevents further steps of primer extension strand displacement and production of multiple copies of the amplification products. The amplification products, or lack thereof, is visualized by gel electrophoresis or other equivalent means.

Factors which contribute to the inhibition of primer hybridization include the size of the hybridizing oligonucleotide and the stringency of the reaction condition. These factors are considered in the design of the composite primer, according to techniques well known and routine in the art.

A composite primer that is complementary to a mutated DNA sequence is also used in combination with a wild type ssDNA target in isothermal amplification method described above. In this case, the primer binds to the target DNA and undergoes extension. However, after treatment with RNase H, more primer cannot bind to the wild type DNA because of nucleotide mismatch and thus, little to no amplification product is made. The amplification products, or lack thereof, is visualized by gel electrophoresis or other equivalent means.

Parallel reactions are also run that include either the nucleic acid sample of interest or reference sample of target nucleic with a wild type sequence. Accumulation of more primer extension products in the former compared to the latter reaction is indicative of the presence of a mutant genotype in the sample of interest. Alternatively, when the composite primer comprises a 5' RNA sequence that is fully complementary to a normal genotype sequence of the test target, amplification of a target sequence of the mutant genotype is

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prevented, and the detection and/or quantitative determination of amplification products is indicative of a normal genotype.

Example 10: Genotyping using isothermal amplification methods and genotype specific probe hybridization

Methods for sequencing by hybridization are described in previous examples. Determination of sequence identity by hybridization of specific probe is particularly advantageous using the isothermal method of the invention insofar as the amplification product generated by the method of the invention is single stranded and readily available to be used in hybridization of specific probes. Probes specific for a defined genotype are designed using methods known in the art. It is possible to determine hybridization criteria which will support selective probe hybridization to amplification products generated by amplification of one genotype and not the other. Sequence variation as small as a single nucleotide can prevent probe hybridization. The following factors are taken into consideration for hybridization criteria: probe length, temperature of the hybridization reaction, and buffer composition, in particular divalent ion concentration. The probes used for the ananlysis may be in solution, or may be attached to a solid surface. Further, the probes may be directly labeled or attached to a member of a specific binding pair and thus, able to specifically bind to another member of the specific binding pair which may be directly or indirectly labeled.

Genomic DNA is isolated from test samples by methods known in the art or as described in the above example. Test DNA is combined with the described amplification components, target-specific chimera primer, and propromoter sequence (such as PTO). The combination is subjected to incubation conditions as described herein to generate single stranded RNA amplification product. Hybridization of the amplification product to genotype specific probes is carried out in solution or solid phase with attached genotype specific probes. Since the products of enhanced isothermal linear amplification methods are single stranded, the products are ideally suited to be attached to a solid phase, such as glass slide, to generate an array of spatially resolved specific probes (i.e., gene chip). Alternatively, the solid phase comprises particles to which specific probes are attached. The detection of probe hybridization to the amplification products is carried out by various

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methods known in the art, for example, disclosed in Sambrook et al. *supra*. The specific probes is labeled, and the change in label spectral properties due to hybridization is detected and recorded by computer algorithms.

Particle association due to hybridization of specific probes to amplification products is also used for the detection of probe hybridization. Labeled amplification products are generated and product hybridization to probes immobilized on solid surfaces is detected and recorded by computer algorithms. The generation of labeled amplification product is carried out by incorporation of labeled rNTPs during the transcription step by substituting of one of the four rNTPs by a rNTP analog, which is labeled. The label is a dye, or a small molecule such as biotin, which is then detected by binding to specific binding entity, such as labeled streptavidin. Methods for detecting probe hybridization on solid surfaces are known in the art.

Example 11: Genotyping by rSSCP (RNA Single Stranded Conformation Polymorphism)

Genotyping is carried out by amplification of the specific target nucleic acid sequence using the methods described herein and by determination of the electrophoretic band pattern of the single stranded RNA product, which reflects the single stranded conformation. The use of SSCP for detection of sequence alteration is widely used. Genotype specific single stranded conformation is determined by subjecting samples to gel or capillary electrophoresis. The generation of single stranded product by amplification of target nucleic acid sequence according to the method of the invention, renders this method particularly suitable for genotype determination by combining the amplification method with rSSCP analysis.

Purified test genomic DNA is combined with components of the amplification method of the invention, as described above, and target specific composite primer and propromoter sequence, such as PTO. The combination is subjected to conditions for isothermal amplification of the target sequence. The reaction mixture containing the amplification product is subjected to either gel electrophoresis or capillary electrophoresis, using instrument and conditions known in the art. The electrophoretic band pattern of the amplification product is determined. The visualization of the oligonucleotide product is

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achieved by inclusion of a dye intercalator. The electrophoretic pattern of the amplification product is compared to that of amplification products generated by amplification of target nucleic acid sequence obtained from cells of known genotype. Any change in the electrophoretic mobility pattern is indicative of sequence variability. The combination of the amplification method of the invention and rSSCP provides a simple method for both the discovery of sequence polymorphism of defined target sequences, and detection of previously defined genotypes. The electrophoretic pattern of known nucleic acid sequences, or defined genotypes, can be predetermined, and the pattern generated by products of amplification of test DNA will be compared to known pattern for genotype determination.

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CLAIMS

What is claimed is:

1. A method for amplifying a polynucleotide sequence complementary to a target polynucleotide sequence comprising:

(a) hybridizing a single stranded DNA template comprising the target sequence with a composite primer, said composite primer comprising an RNA portion and a 3' DNA portion;

(b) optionally hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the template which is 5' with respect to hybridization of the composite primer to the template;

(c) extending the composite primer with DNA polymerase;

(d) cleaving the RNA portion of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer can hybridize to the template and repeat primer extension by strand displacement,

whereby multiple copies of the complementary sequence of the target sequence are produced.

2. A method for amplifying a target polynucleotide sequence comprising:

(a) hybridizing a single stranded DNA template comprising the target sequence with a composite primer, said composite primer comprising an RNA portion and a 3' DNA portion;

(b) hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the template which is 5' with respect to hybridization of the composite primer to the template;

(c) extending the composite primer with DNA polymerase;

(d) cleaving the RNA portion of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer can hybridize to the template and repeat primer extension by strand displacement to produce displaced primer extension product;

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(e) hybridizing a polynucleotide comprising a propromoter and a region which hybridizes to the displaced primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences complementary to the displaced primer extension products, whereby multiple copies of the target sequence are produced.

3. The method of claims 1 or 2, wherein the RNA portion of the composite primer is 5' with respect to the 3' DNA portion.

4. The method of claim 3, wherein the 5' RNA portion is adjacent to the 3' DNA portion.

5. The method of claims 1 or 2, wherein a plurality of composite primers are used.

6. The method of claims 1 or 2, wherein the polynucleotide comprising a termination sequence is a template switch oliognucleotide (TSO).

7. The method of claim 6, wherein the TSO comprises a modification in the region which hybridizes to the template, wherein, under a given set of conditions, the TSO binds more tightly to the region as compared to a TSO without the modification.

8. The method of claims 1 or 2, wherein the polynucleotide comprising a termination sequence is a blocking sequence.

9. The method of claim 8, wherein the blocking sequence comprises a modification in the region which hybridizes to the template, wherein, under a given set of conditions, the blocking sequence binds more tightly to the region as compared to a blocking sequence without the modification.

10. The method of claims 1 or 2, wherein the enzyme that cleaves RNA is RNaseH.

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11. The method of claim 2, wherein the polynucleotide comprising a propromoter and region which hybridizes to the displaced primer extension product is a template switch oligonucleotide (TSO).

12. The method of claim 2, wherein the polynucleotide comprising the propromoter comprises a region at the 3' end which hybridizes to the displaced primer extension product, whereby DNA polymerase extension of displaced primer extension product produces a double stranded promoter from which transcription occurs.

13. The method of claim 12, wherein the polynucleotide comprising the propromoter is a propromoter template oligonucleotide (PTO).

14. The method of claims 1 or 2, wherein steps (a) and (b) are performed in either order.

15. The method of claims 1 or 2, wherein steps (a) and (b) are performed simultaneously.

16. The method of claims 1 or 2, wherein steps (a), (b) and (c) are performed simultaneously.

17. The method of claims 1 or 2, wherein steps (a) and (b) are performed before step (c).

18. The method of claims 1 or 2, wherein all steps are performed simultaneously.

19. A method of sequencing a target nucleotide sequence comprising:

(a) hybridizing a single stranded DNA template comprising the target sequence with a composite primer, said composite primer comprising an RNA portion and a 3' DNA portion;

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(b) optionally hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the template which is 5' with respect to hybridization of the composite primer to the template;

(c) extending the composite primer to a termination site with DNA polymerase and a mixture of dNTPs and dNTP analogs, such that primer extension is terminated upon incorporation of a dNTP analog;

(d) cleaving the RNA portion of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer can hybridize to the template and repeat primer extension by strand displacement, whereby multiple copies of the complementary sequence of the target sequence are produced of varying lengths;

(e) analyzing the product of steps (a) through (d) to determine sequence.

20. A method of sequencing a target nucleotide sequence comprising

(a) hybridizing a single stranded DNA template comprising the target sequence with a composite primer, said composite primer comprising an RNA portion and a 3' DNA portion;

(b) hybridizing a polynucleotide comprising a termination polynucleotide sequence to a region of the template which is 5' with respect to hybridization of the composite primer to the template;

(c) extending the composite primer with DNA polymerase;

(d) cleaving the RNA portion of the annealed composite primer with an enzyme that cleaves RNA from an RNA/DNA hybrid such that another composite primer can hybridize to the template and repeat primer extension by strand displacement to produce displaced primer extension product;

(e) hybridizing a polynucleotide comprising a propromoter and a region which hybridizes to the displaced primer extension product under conditions such that transcription occurs from the extension product by RNA polymerase, using a mixture of rNTPs and rNTP analogs, such that RNA transcripts are produced comprising sequences complementary to the displaced primer extension products, and such that transcription is

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terminated upon incorporation of an rNTP analog, whereby multiple copies of the target sequence are produced of varying lengths;

(f) analyzing the product of steps (a) through (e) to determine sequence.

21. The method of claim 19 or 20, wherein the RNA portion of the composite primer is 5' with respect to the 3' DNA portion.

22. The method of claim 21, wherein the 5' RNA portion is adjacent to the 3' DNA portion.

23. A method of characterizing a sequence of interest in a target polynucleotide, said method comprising

conducting the method of claims 1 or 2 wherein the sequence of an RNA portion of the composite primer is known, and wherein

(a) production of detectably fewer amplification products from the template as compared to the amount of amplification products from a reference template which comprises a region complementary to the RNA portion of the composite primer indicates that the target polynucleotide does not comprise a sequence complementary to the RNA portion of the composite primer and is a sequence variant with respect to the sequence complementary to the RNA portion of the composite primer; or

(b) production of detectably more amplification products from the template as compared to the amount of amplification products from a reference template which does not comprise a region which is complementary to the RNA portion of the composite primer indicates that the target polynucleotide comprises a sequence complementary to the RNA portion of the composite primer and is not a sequence variant with respect to the sequence complementary to the RNA portion of the composite primer.

24. The method of claim 23, wherein the sequence of an RNA portion of the composite primer comprises a wild type sequence, and the sequence of interest is characterized in determining the presence or absence of the wild type sequence.

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25. The method of claim 23, wherein the sequence of an RNA portion of the composite primer comprises a mutant sequence, and the sequence of interest is characterized in determining the presence or absence of the mutant sequence.

26. The method of claim 23, wherein the sequence of an RNA portion of the composite primer comprises an allelic sequence, and the sequence of interest in characterized in determining the presence or absence of the allelic sequence.

27. A method of detecting a mutation in a target polynucleotide, comprising (a) conducting the method of claims 1 or 2; and (b) analyzing the amplified products of the method for single stranded conformation, wherein a difference in conformation as compared to a reference single stranded polynucleotide indicates a mutation in the target polynucleotide.

28. The method of claims 23, wherein the RNA portion of the composite primer is 5' with respect to the 3' DNA portion.

29. The method of claim 28, wherein the 5' RNA portion is adjacent to the 3' DNA portion.

30. A method of producing a microarray, comprising (a) conducting the amplification method of claims 1 or 2; and (b) attaching the amplified products onto a solid substrate to make a microarray of the amplified products.

31. A composition comprising a composite primer, said composite primer comprising a 3' DNA portion and a 5' RNA portion.

32. The composition of claim 31, wherein the 5' RNA portion is adjacent to the 3' DNA portion.

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33. The composition of claim 31, wherein the 5' RNA portion is about 5 to about 20 nucleotides and the 3' DNA portion is about 5 to about 15 nucleotides.

34. A composition comprising a TSO, wherein the TSO comprises a modification in the region which hybridizes to the template, wherein, under a given set of conditions, the TSO binds more tightly to the region as compared to a TSO without the modification.

35. A composition comprising the composite primer of claim 31 and the TSO of claim 34.

36. A composition comprising the composite primer of claim 31 and a blocking sequence.

37. A composition comprising the composite primer of claim 31 and a propromoter template oligonucleotide (PTO).

38. A composition comprising a complex of (a) a template strand; and (b) a composite primer, said composite primer comprising a 3' DNA portion and an RNA portion.

39. The composition of claim 38, wherein the RNA portion is 5' and adjacent to the 3' DNA portion.

40. The composition of claim 39, wherein the complex further comprises a termination sequence.

41. The composition of claim 40, wherein the termination sequence is a TSO.

42. The composition of claim 40, wherein the termination sequence is a blocking sequence.

IDT Exhibit 1020.8550

43. A reaction mixture comprising (a) a polynucleotide template; (b) a composite primer comprising a 3' DNA portion and an RNA portion; and (c) DNA polymerase.

44. The reaction mixture of claim 43, wherein the composite primer comprises a 5' RNA portion which is adjacent to the 3' DNA portion.

45. The reaction mixture of claim 44, further comprising an enzyme which cleaves RNA from an RNA/DNA hybrid.

46. The reaction mixture of claim 45, wherein the enzyme is RNaseH.

47. The reaction mixture of claim 44, further comprising a polynucleotide comprising a termination polynucleotide sequence.

48. The reaction mixture of claim 47, further comprising a polynucleotide comprising a propromoter.

49. The reaction mixture of claim 48, wherein the polynucleotide comprising a propromoter is a TSO.

50. The reaction mixture of claim 48, wherein the polynucleotide comprising a propromoter is a propromoter template oligonucleotide (PTO).

51. A kit for amplification of a target polynucleotide sequence, comprising a composite primer comprising a 3' DNA portion and an RNA portion.

52. The kit of claim 51, wherein the RNA portion is 5' with respect to the 3' DNA portion.

53. The kit of claim 52, wherein the 5' RNA portion is adjacent to the 3' DNA portion.

IDT Exhibit 1020.8551

54. The kit of claim 51, further comprising a polynucleotide comprising a termination polynucleotide sequence.

55. The kit of claim 54, wherein the termination polynucleotide sequence is a TSO.

56. The kit of claim 54, wherein the termination polynucleotide sequence is a blocker sequence.

57. The kit of claim 51, further comprising a polynucleotide comprising a propromoter.

58. The kit of claim 57, wherein the polynucleotide comprising a propromoter is a TSO.

59. The kit of claim 57, wherein the polynucleotide comprising a propromoter is a PTO.

60. The kit of claim 51, further comprising an enzyme which cleaves RNA from an RNA/DNA hybrid.

61. The kit of claim 60, wherein the enzyme is RNaseH.

62. A system for amplifying a target polynucleotide sequence or its complement, comprising (a) a composite primer comprising a 3' DNA portion and an RNA portion; (b) DNA polymerase; and (c) an enzyme which cleaves RNA from an RNA/DNA hybrid.

63. The system of claim 62, wherein the enzyme is RNaseH.

64. The system of claims 62 or 63, wherein the composite primer comprises a 5' RNA portion which is adjacent to the 3' DNA portion.

IDT Exhibit 1020.8552

FIGURE 1A

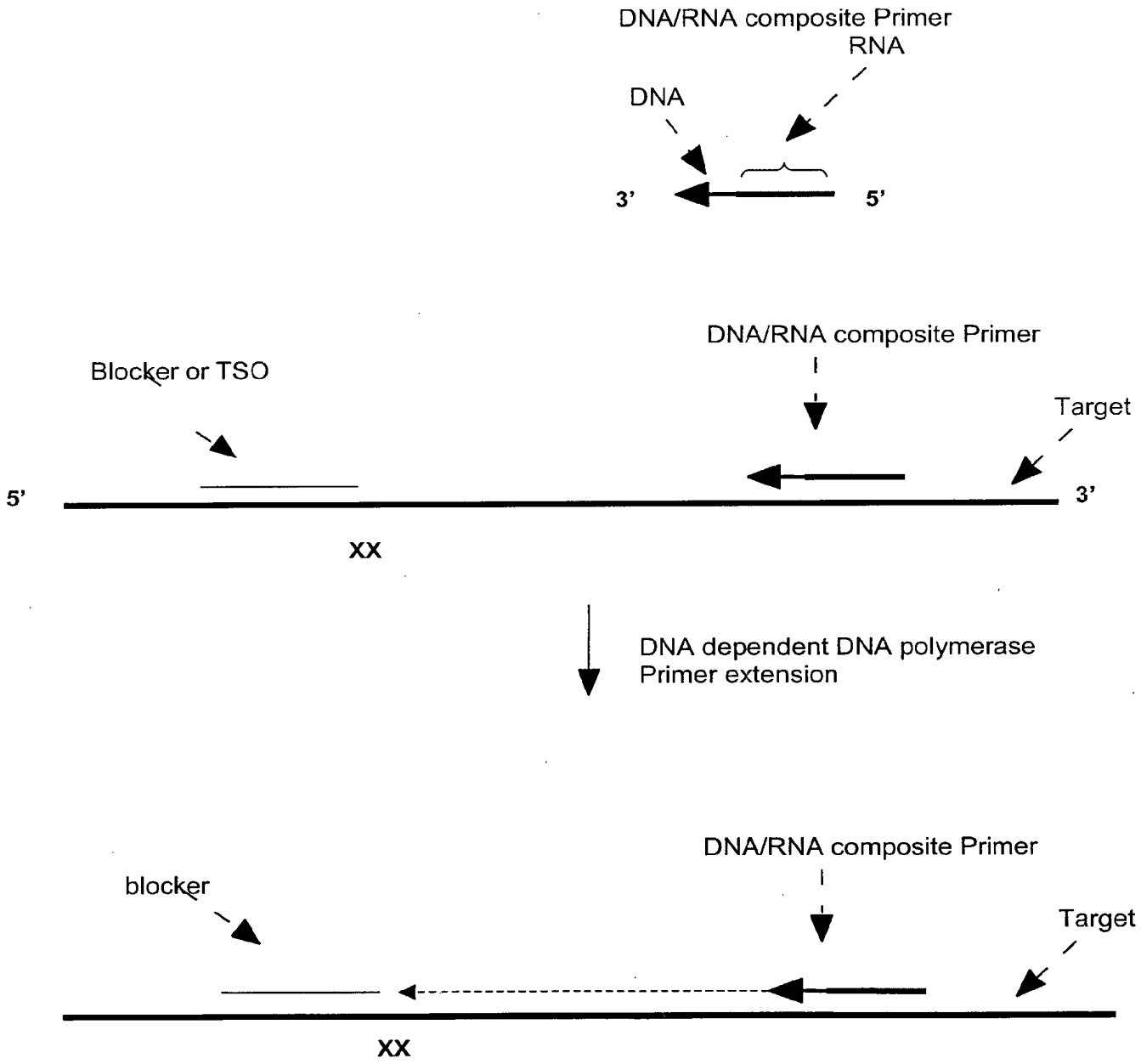

DNA/RNA composite Primer
RNA
DNA
3'
5'
DNA/RNA composite Primer
Blocker or TSO
Target
5'
3'
XX
DNA dependent DNA polymerase
Primer extension
DNA/RNA composite Primer
blocker
Target
XX

FIGURE 1B

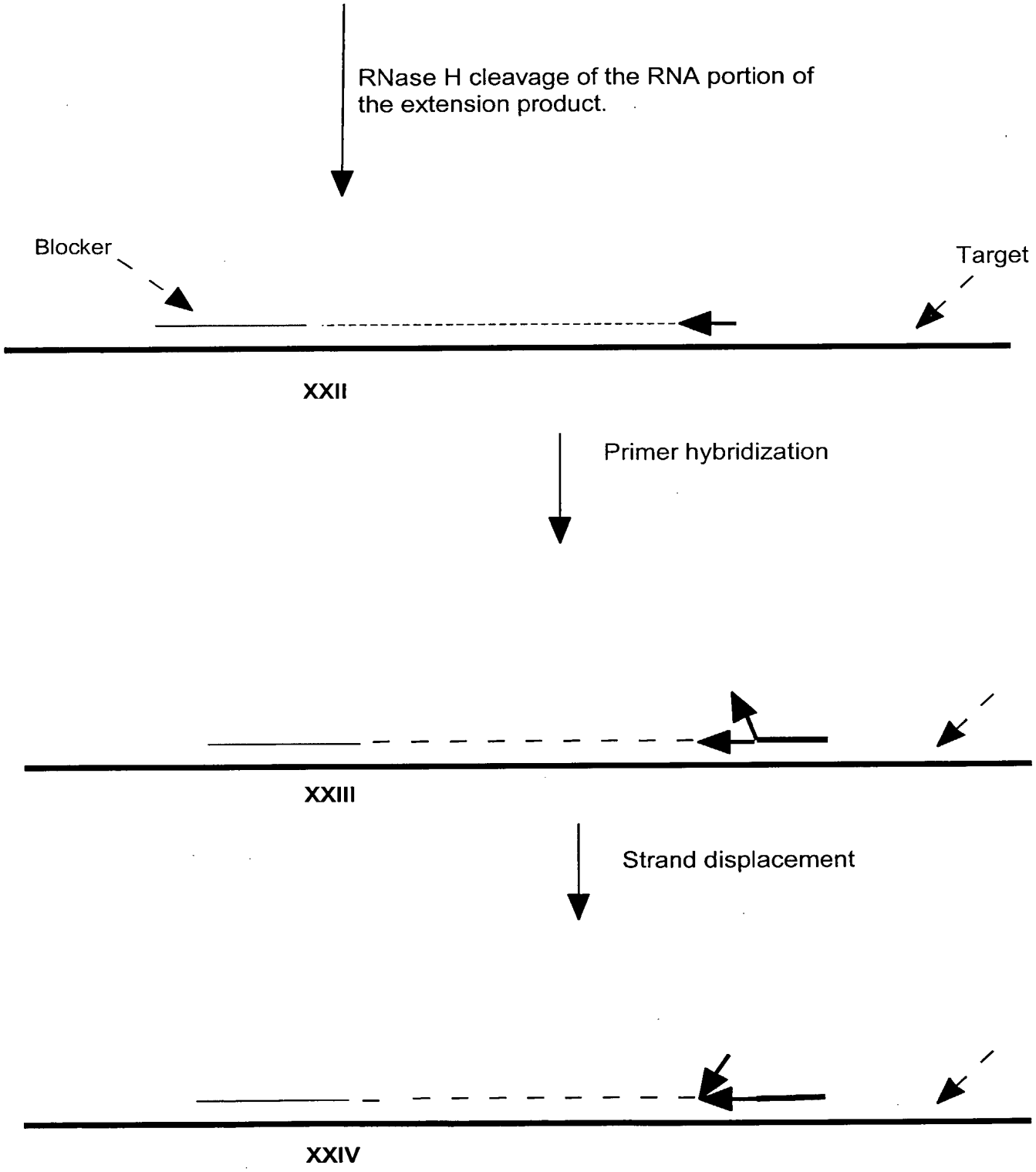

RNase H cleavage of the RNA portion of the extension product.
Blocker
Target
XXII
Primer hybridization
XXIII
Strand displacement
XXIV

FIGURE 1C

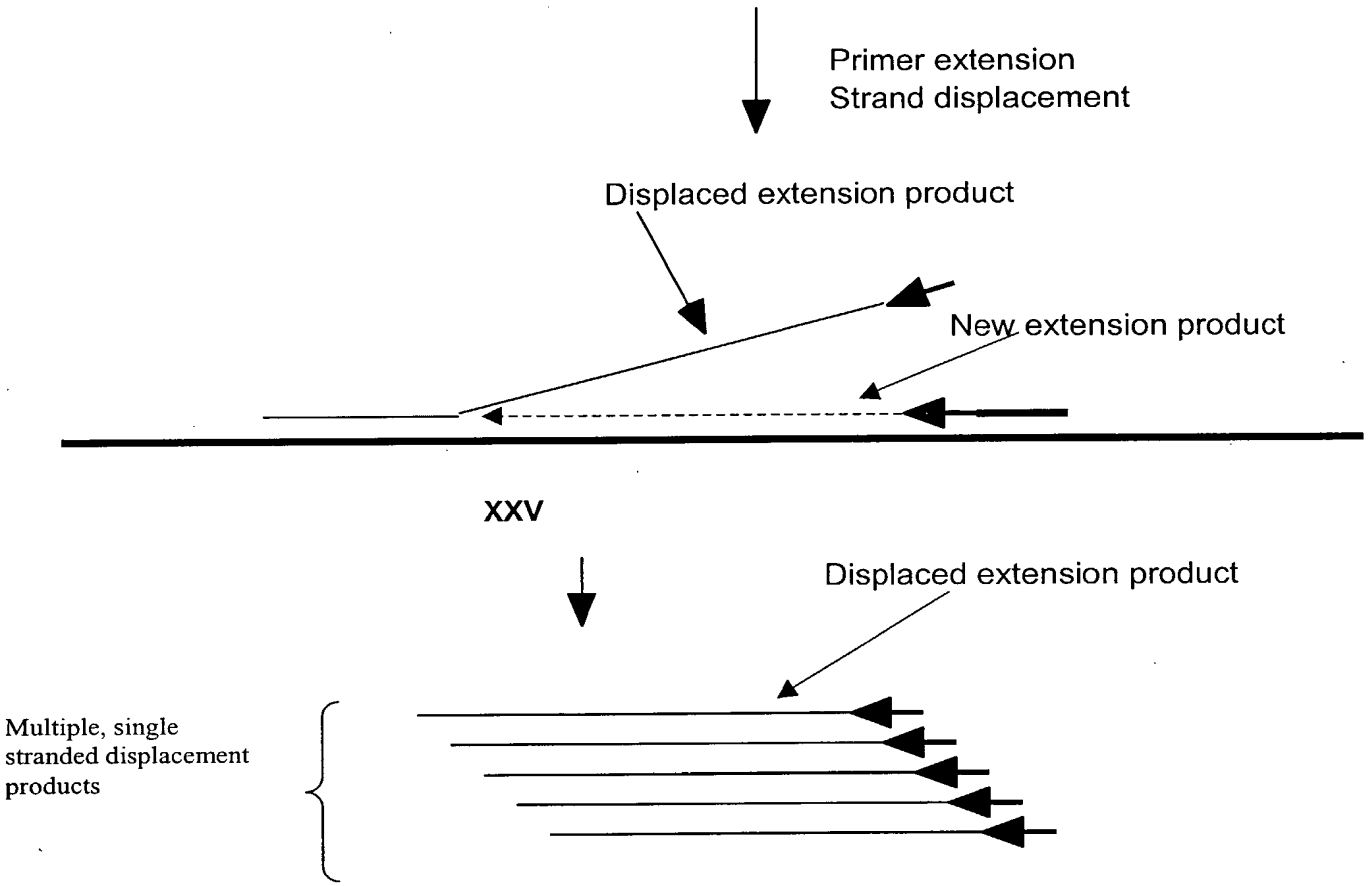

Primer extension
Strand displacement
Displaced extension product
New extension product
XXV
Displaced extension product
Multiple, single stranded displacement products

FIGURE 2A

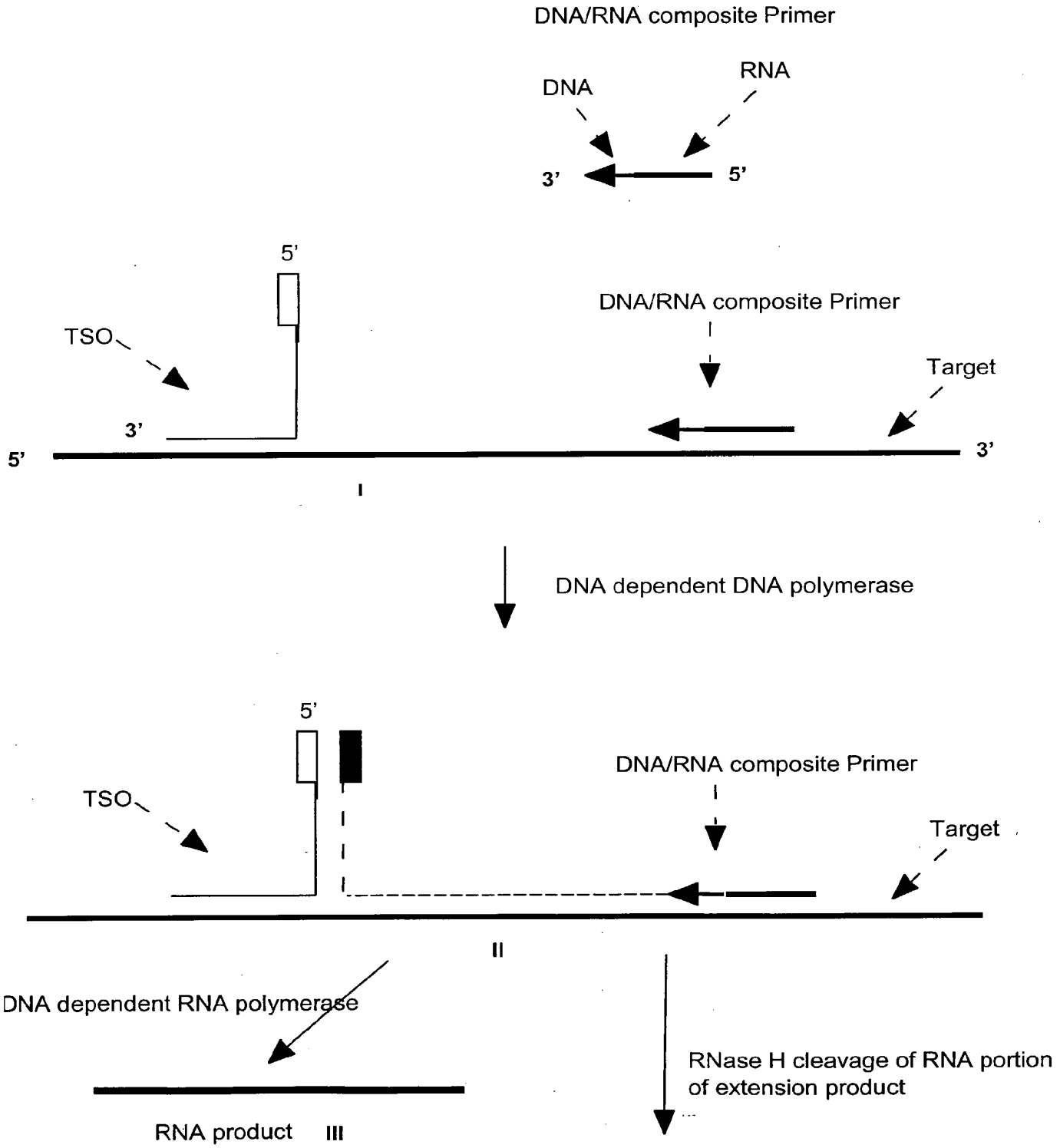

DNA/RNA composite Primer
DNA
RNA
3'
5'
5'
TSO
3'
DNA/RNA composite Primer
Target
5'
3'
I
DNA dependent DNA polymerase
5'
TSO
DNA/RNA composite Primer
Target
II
DNA dependent RNA polymerase
RNase H cleavage of RNA portion
of extension product
RNA product III

FIGURE 2B

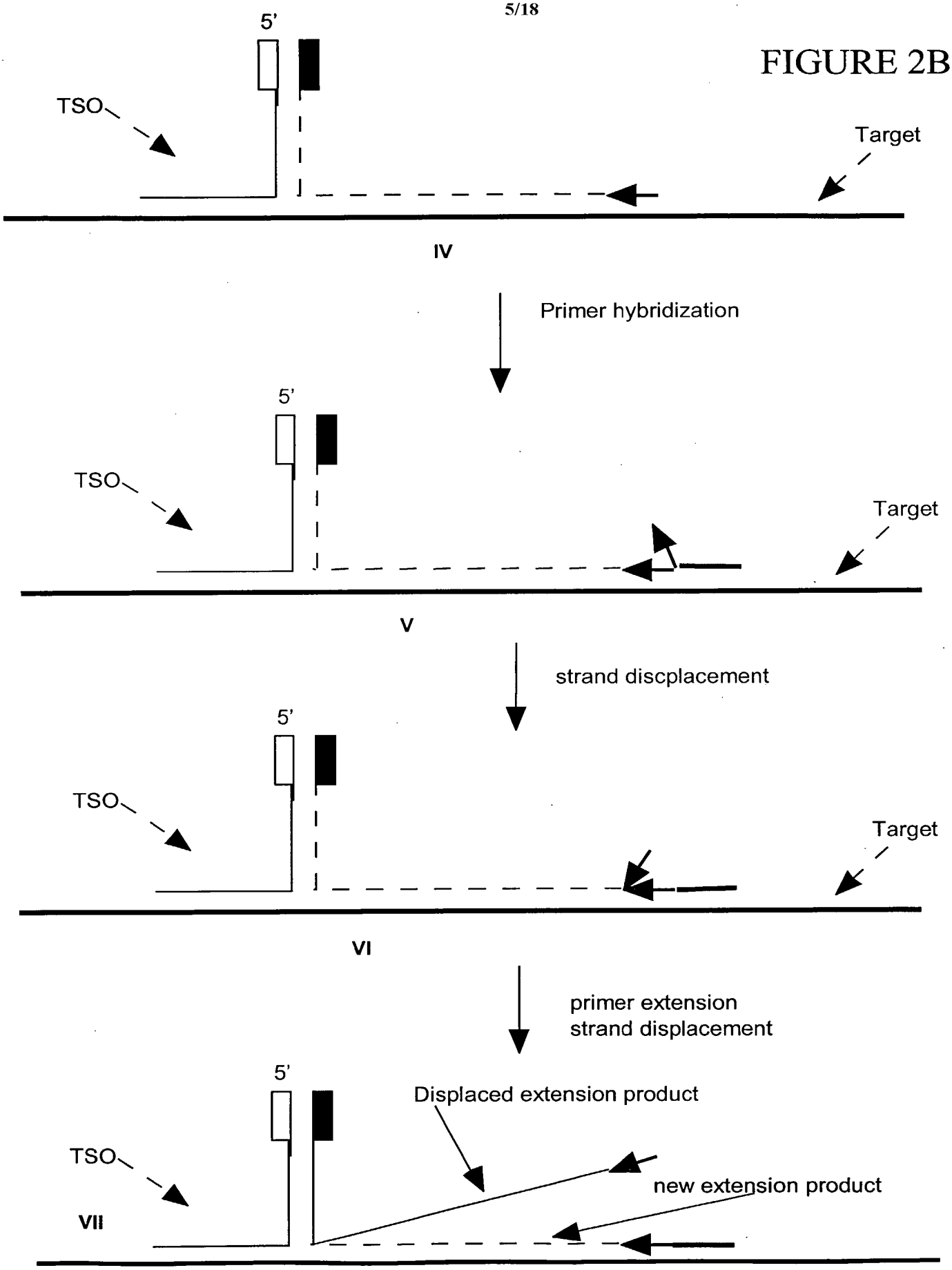

5'
TSO
Target
IV
Primer hybridization
5'
TSO
Target
V
strand discplacement
5'
TSO
Target
VI
primer extension
strand displacement
5'
Displaced extension product
TSO
new extension product
VII

FIGURE 2C

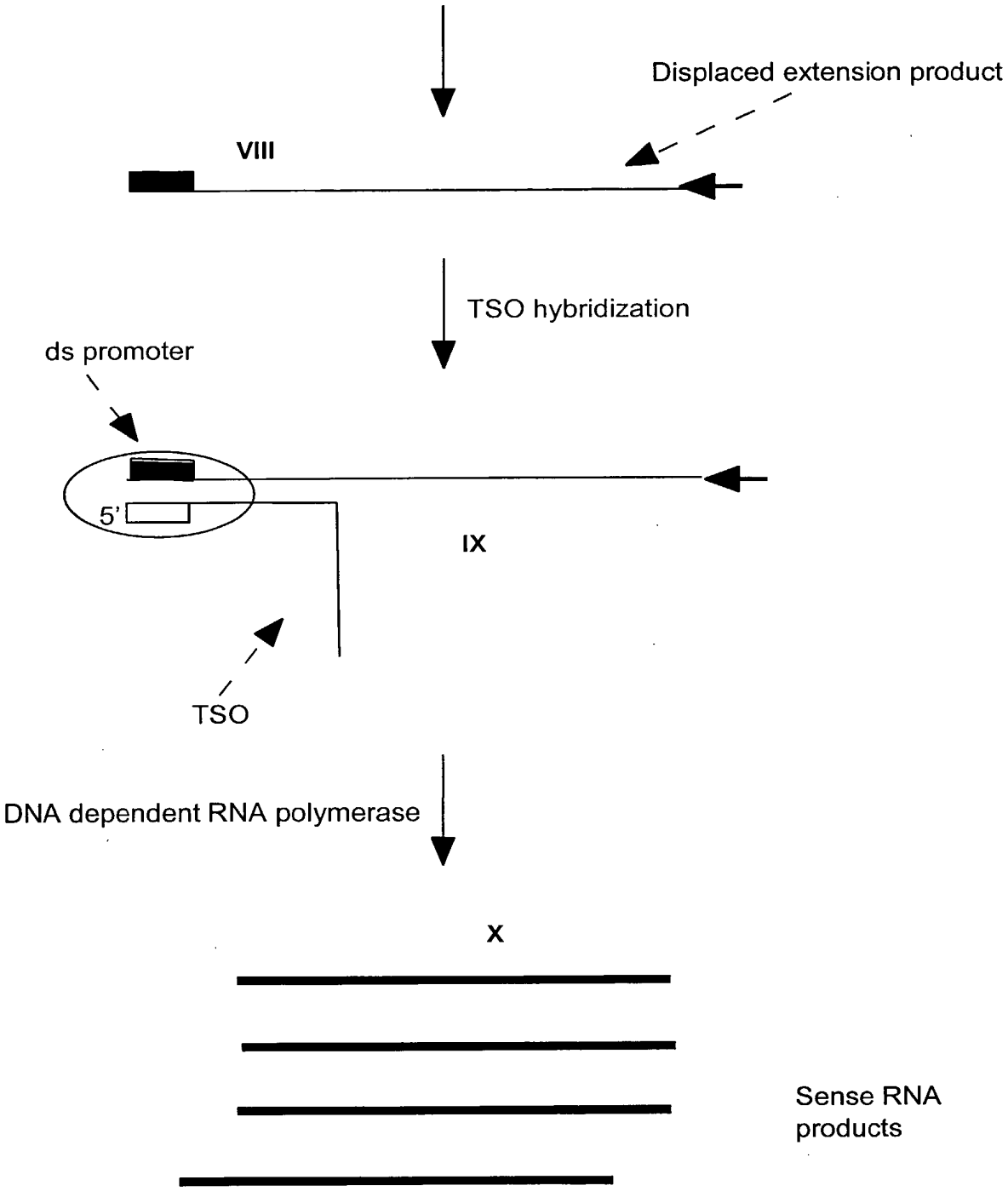

Displaced extension product
VIII
TSO hybridization
ds promoter
5'
IX
TSO
DNA dependent RNA polymerase
X
Sense RNA products

FIGURE 3A

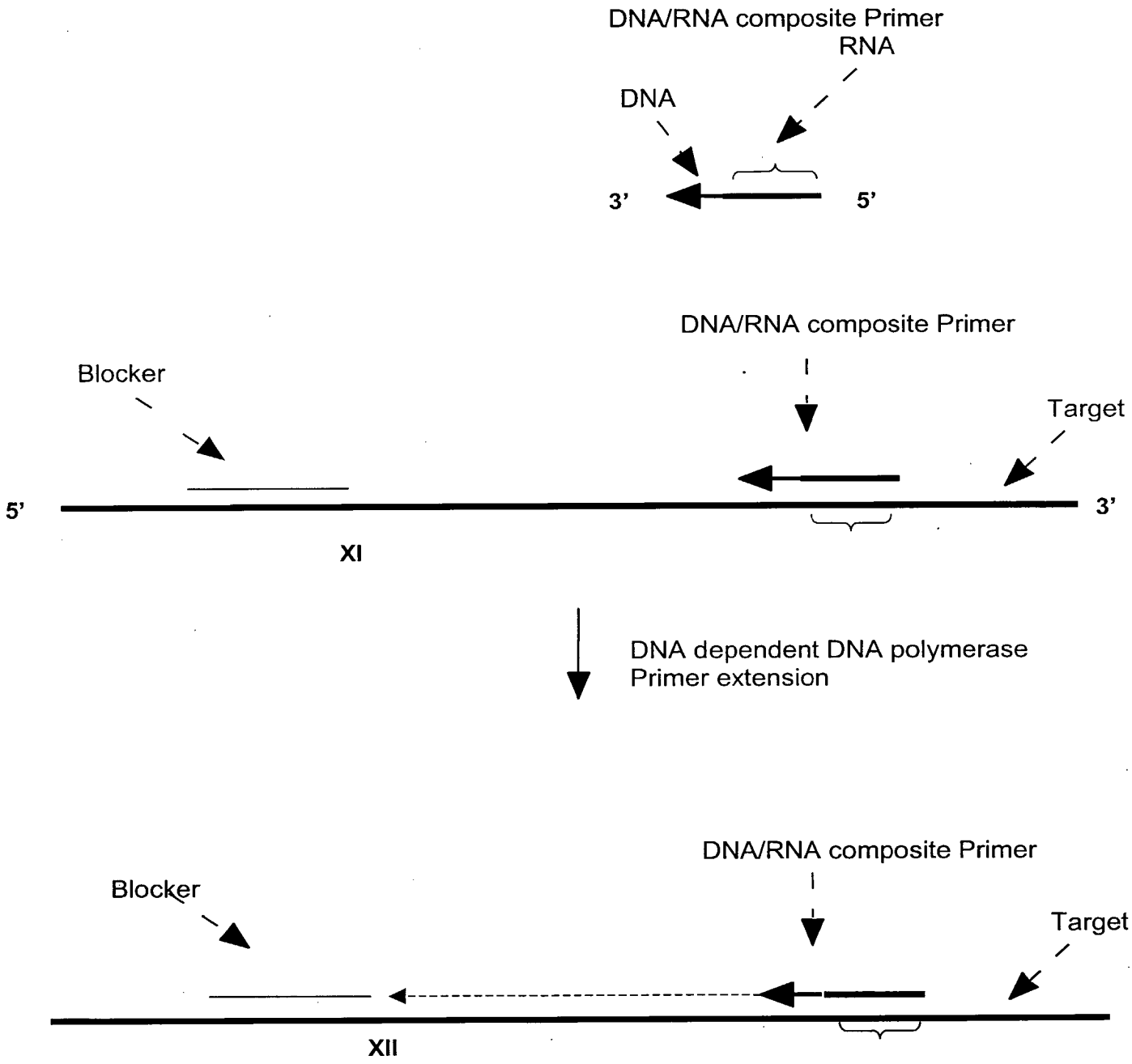

DNA/RNA composite Primer
RNA
DNA
3'
5'
DNA/RNA composite Primer
Blocker
Target
5'
3'
XI
DNA dependent DNA polymerase
Primer extension
DNA/RNA composite Primer
Blocker
Target
XII

FIGURE 3B

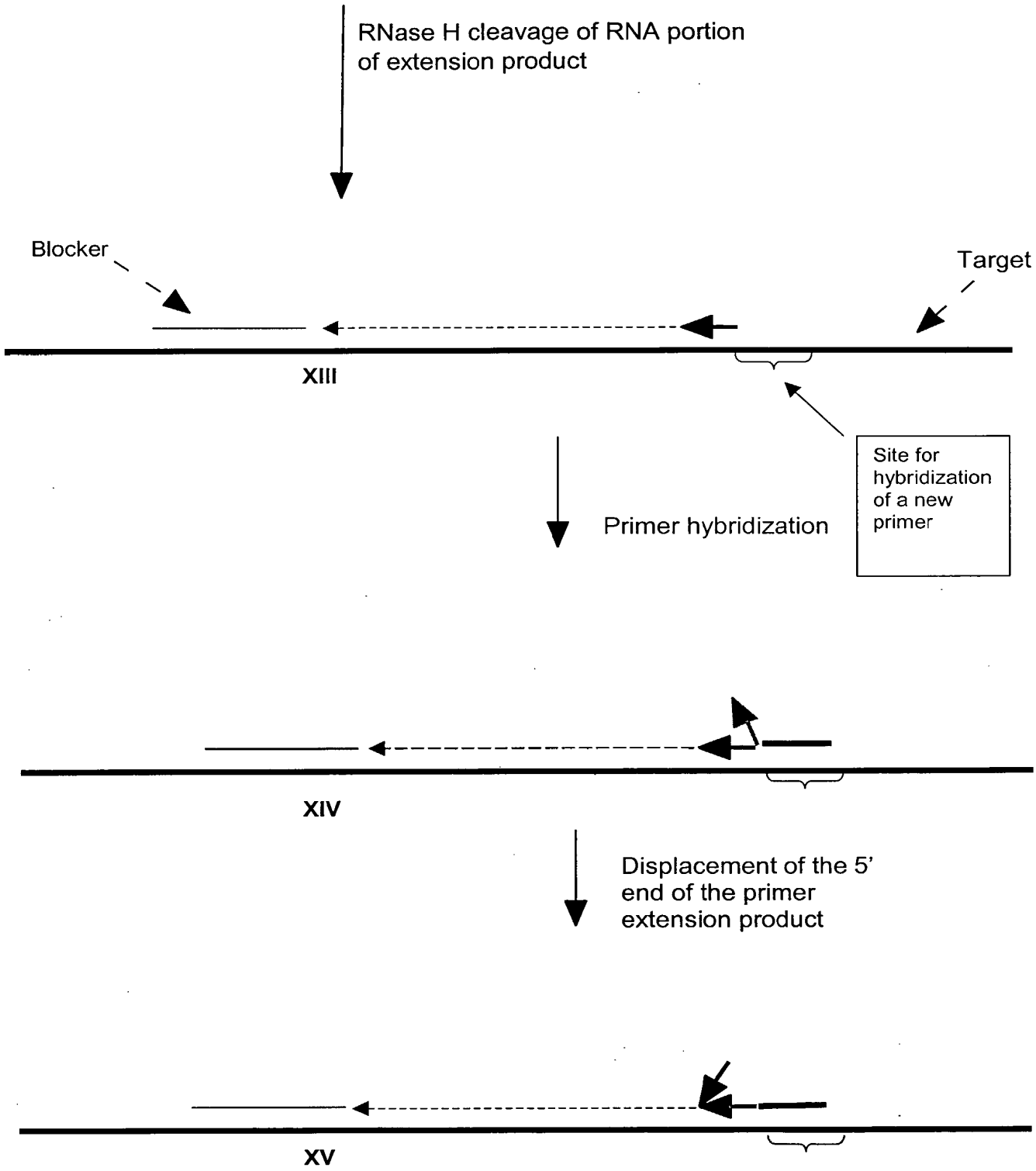

RNase H cleavage of RNA portion of extension product
Blocker
Target
XIII
Site for hybridization of a new primer
Primer hybridization
XIV
Displacement of the 5' end of the primer extension product
XV

FIGURE 3C

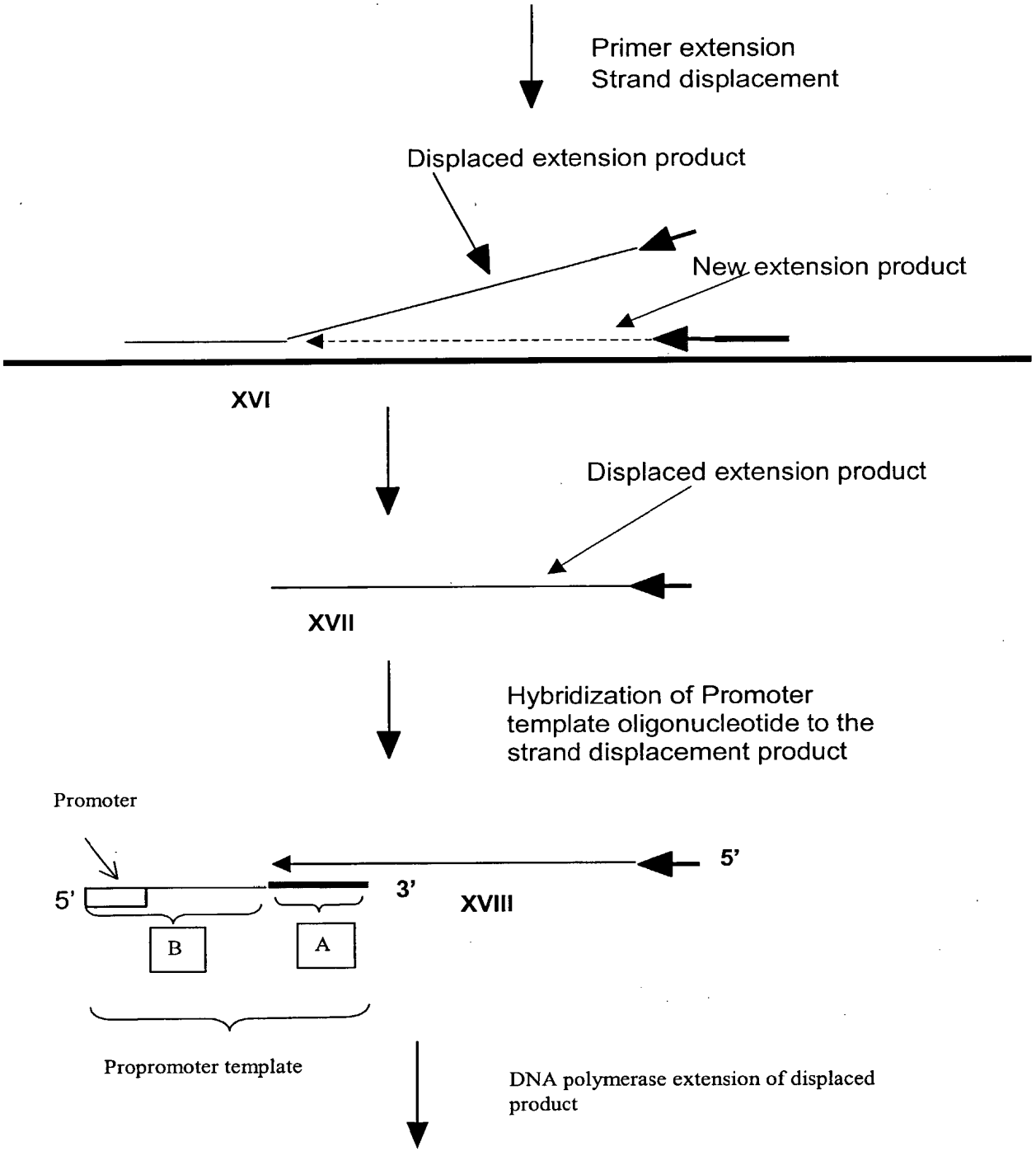

Primer extension
Strand displacement
Displaced extension product
New extension product
XVI
Displaced extension product
XVII
Hybridization of Promoter template oligonucleotide to the strand displacement product
Promoter
5'
3'
5'
XVIII
B
A
Propromoter template
DNA polymerase extension of displaced product

FIGURE 3D

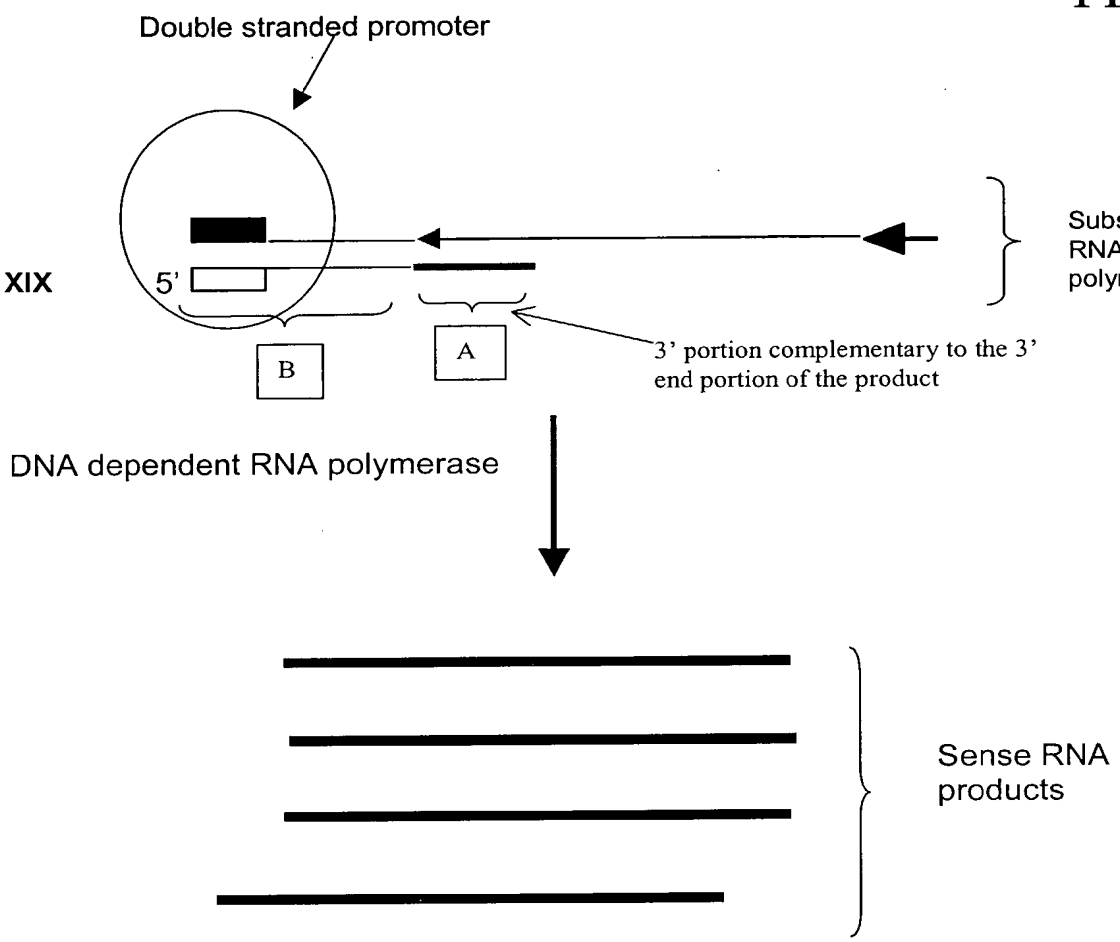

Double stranded promoter
XIX
5'
B
A
Substrate for RNA polymerase
3' portion complementary to the 3' end portion of the product
DNA dependent RNA polymerase
Sense RNA products

FIGURE 4A

X denotes a mutation on the target DNA at a site complementary to the 5' RNA portion of the primer.
As shown, amplification of the target NA is blocked when a mutation is present.

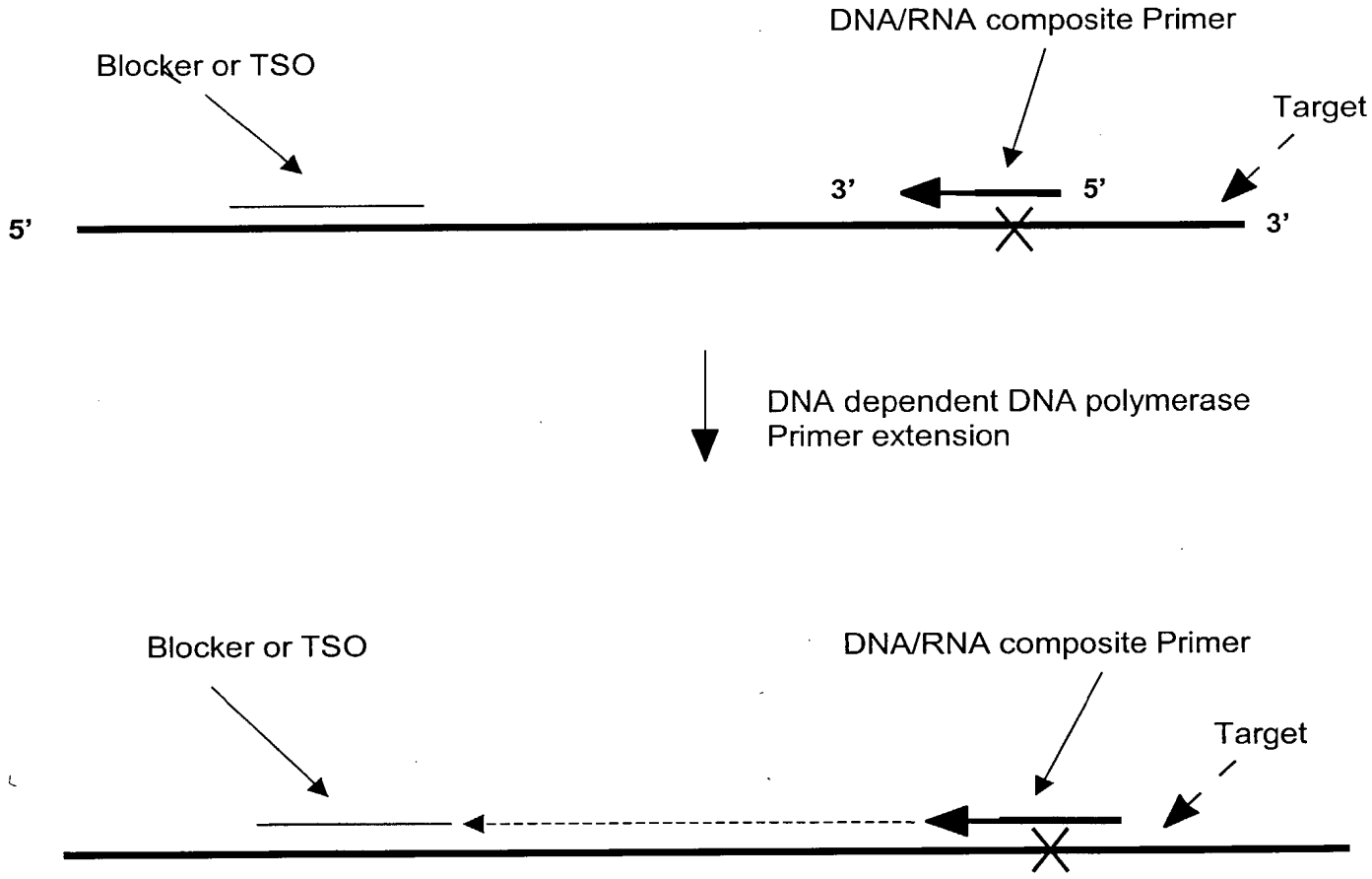


FIGURE 4B

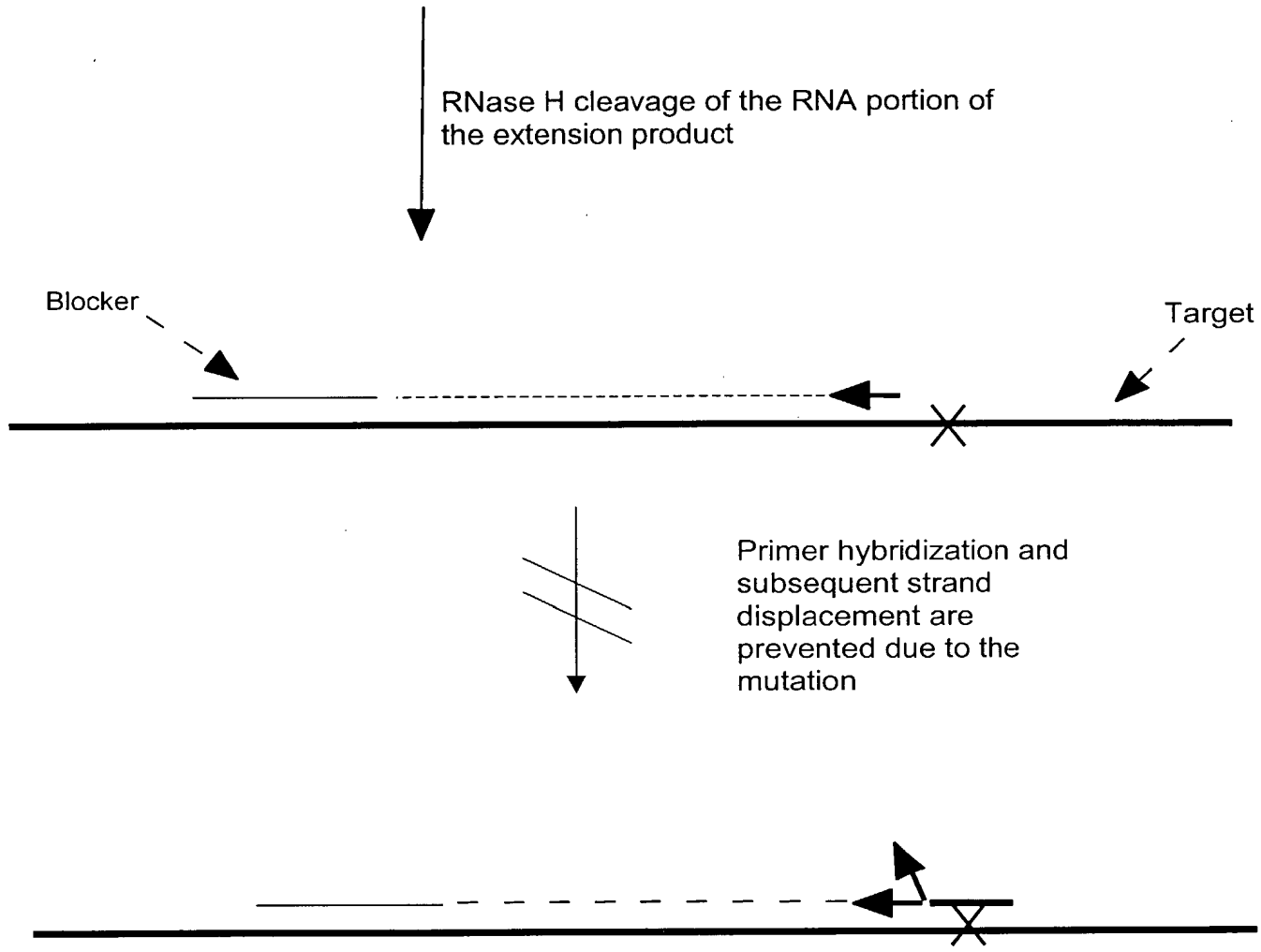
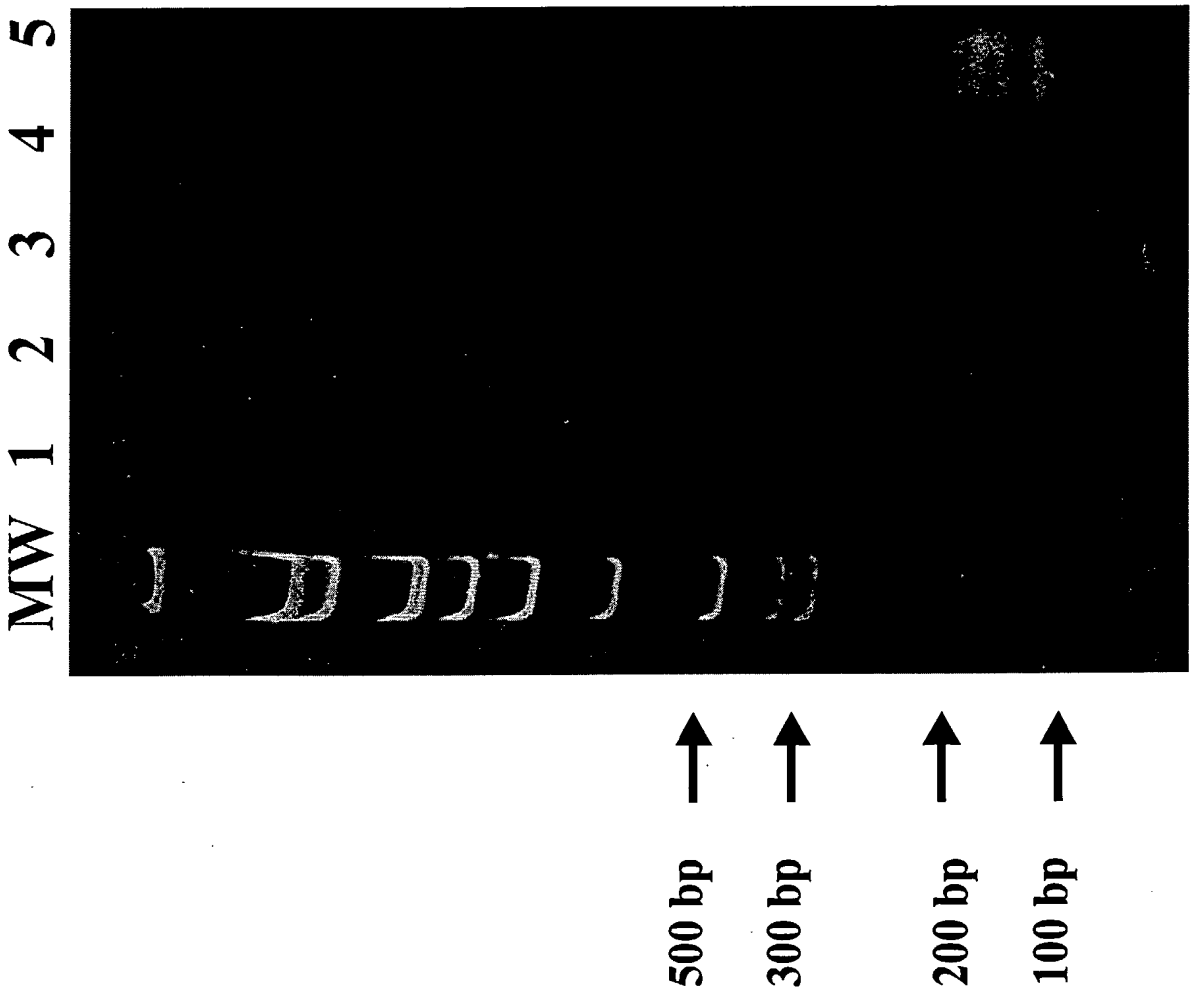

RNase H cleavage of the RNA portion of the extension product
Blocker
Target
Primer hybridization and subsequent strand displacement are prevented due to the mutation

Figure 5



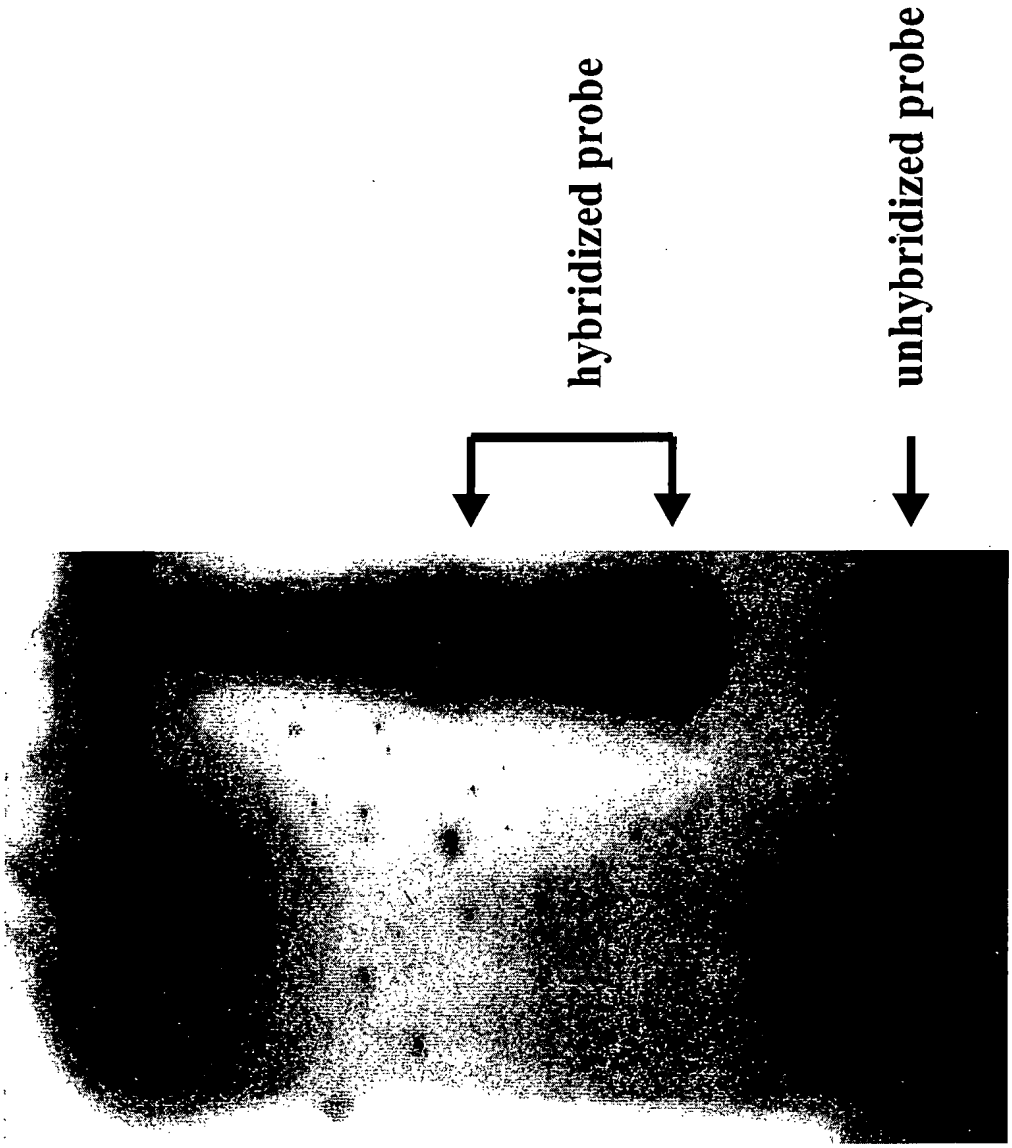


Figure 6

Figure 7

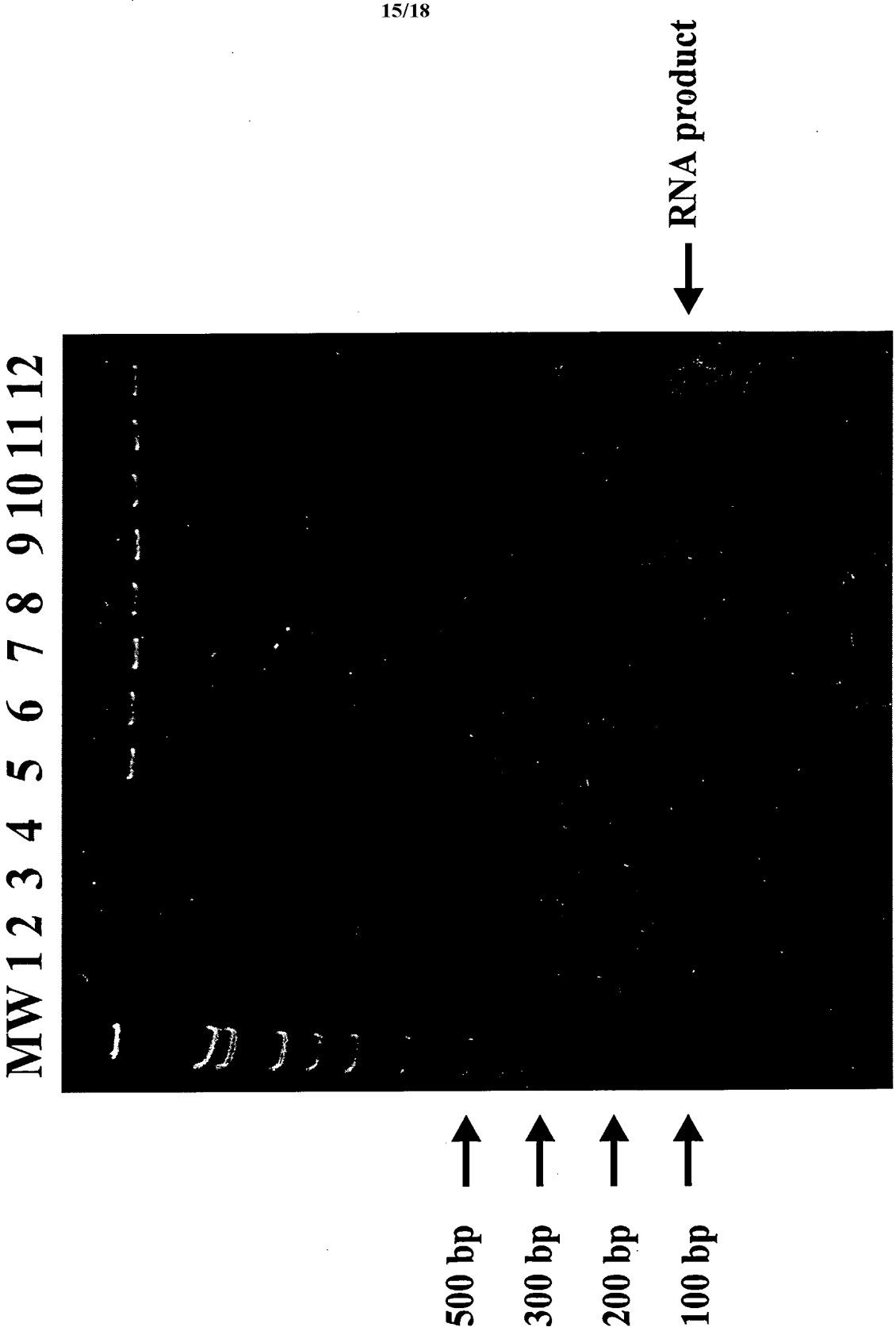


Figure 8

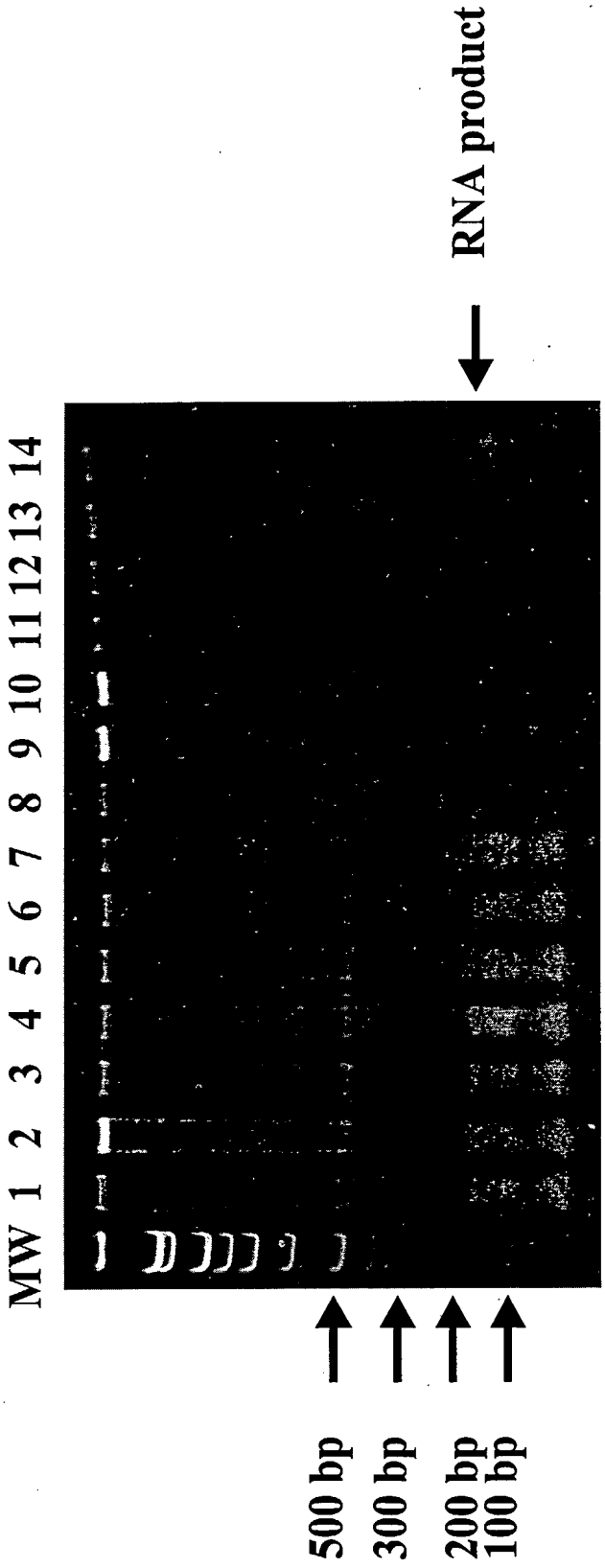

RNA product
MW 1 2 3 4 5 6 7 8 9 10 11 12 13 14
500 bp
300 bp
200 bp
100 bp

Figure 9

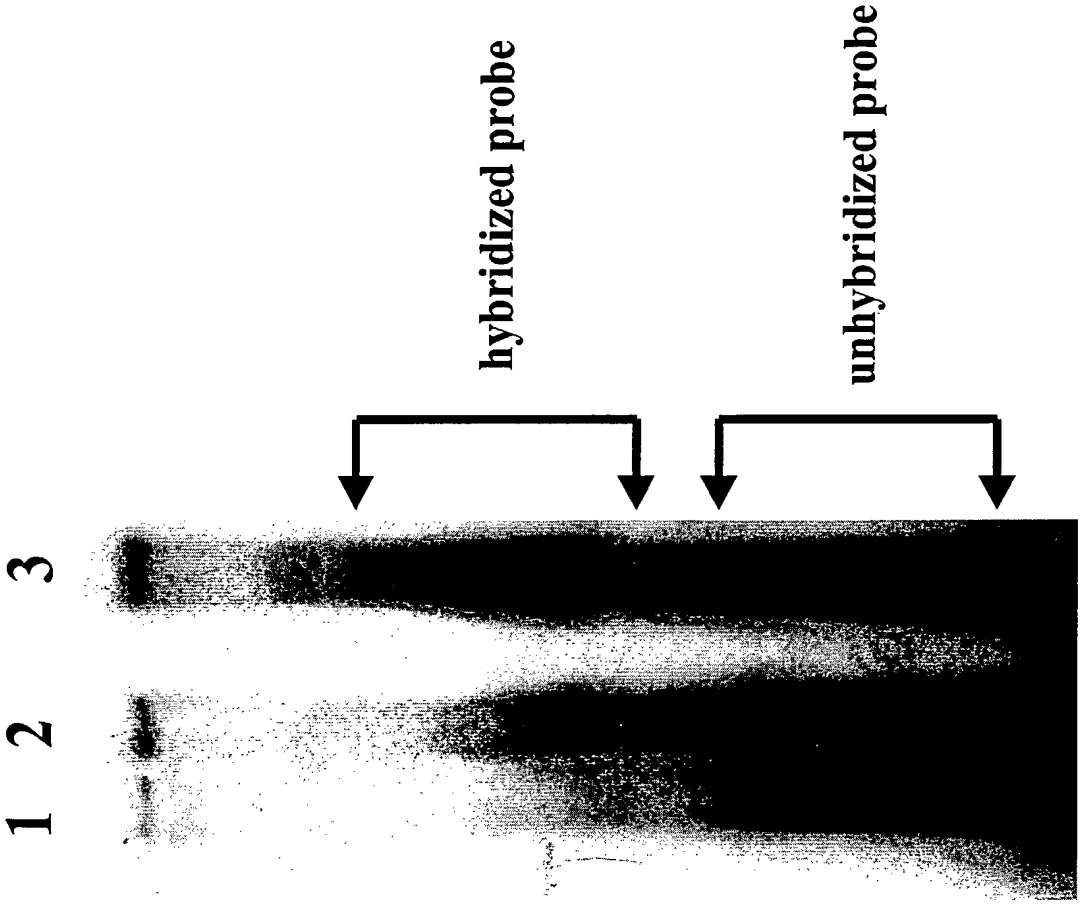
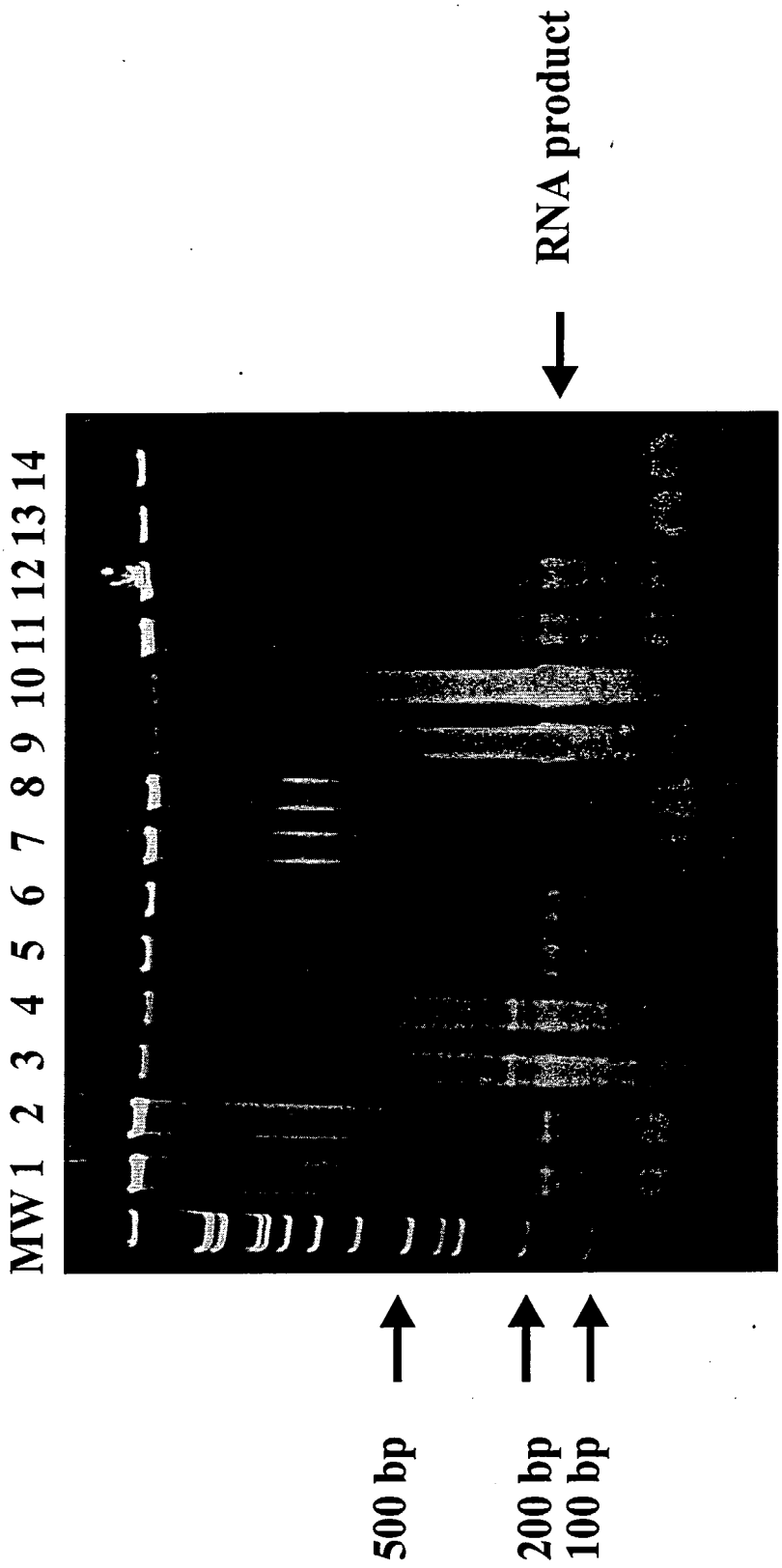


Figure 10



SEQUENCE LISTING

<110> Kurn, N.

<120> METHODS AND COMPOSITIONS FOR LINEAR
ISOTHERMAL AMPLIFICATION OF POLYNUCLEOTIDE SEQUENCES

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IDT Exhibit 1020.8572

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IDT Exhibit 1020.8573

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IDT Exhibit 1020.8574

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IDT Exhibit 1020.8575

(19)中华人民共和国国家知识产权局

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(51)Int.Cl.
G01G 3/16(2006.01)

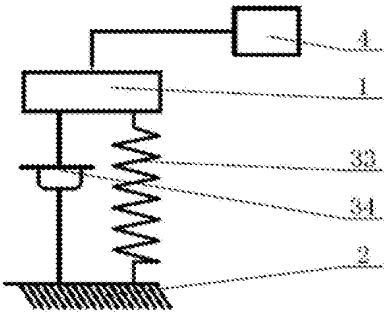
权利要求书1页 说明书2页 附图1页

(54)发明名称

利用共振原理测量DNA分子质量的方法

(57)摘要

本发明公开了一种利用共振原理测量DNA分子质量的方法，属于有机大分子质量的检测与称量领域。它包括激振器、铰支座A、铰支座B、两端分别装设于铰支座A和铰支座B上的微简支梁、装设于微简支梁上的DNA分子吸附层、待测量的DNA分子、装设于微简支梁上的振幅接收器；通过激振器激励微简支梁自由振动，振幅接收器接受相应的振幅和振动频率，从而计算出DNA分子的质量。本发明是一种原理简单、测量精度更高的共振原理测量DNA分子质量的方法。



CN 105890722 A

IDT Exhibit 1020.8576

1.利用共振原理测量DNA分子质量的方法，其特征在于：包括激振器(4)、铰支座A(21)、铰支座B(22)、两端分别装设于所述铰支座A(21)和所述铰支座B(22)上的微简支梁(32)、装设于所述微简支梁(32)上的DNA分子吸附层(31)、待测量的DNA分子(1)、装设于所述微简支梁(32)上的振幅接收器(5)。

2.根据权利要求1所述的利用共振原理测量DNA分子质量的方法，其特指在于由下述步骤组成：

(a)采用所述激振器(4)作用所述微简支梁(32)，使微简支梁(32)发生自由振动；采用所述振幅接收器(5)接收微简支梁(32)的振幅，并记录下最大振幅与相应的振动频率；确定微简支梁(32)无DNA分子时的固有频率f0；

(b)将待测量的DNA分子(1)放置于所述DNA分子吸附层(31)上，采用所述激振器(4)作用所述微简支梁(32)，使微简支梁(32)发生自由振动；采用所述振幅接收器(5)接收微简支梁(32)的振幅，并记录下最大振幅与相应的振动频率；采用所述振幅接收器(5)接收微简支梁(32)的振幅，并记录下最大振幅与相应的振动频率；确定有DNA分子(1)时微简支梁(32)的固有频率f1；

(c)系统的等效弹簧(33)的等效刚度为k，等效阻尼器(34)的等效阻尼为η，建立DNA分子(1)的共振模型；并利用公式$m=(K/6.28)^2\times(1/f1-1/f0)$计算出DNA分子(1)的质量。

IDT Exhibit 1020.8577

利用共振原理测量DNA分子质量的方法

技术领域

[0001] 本发明主要涉及有机大分子质量的检测与称量领域，特指一种利用共振原理测量DNA分子质量的方法。

背景技术

[0002] DNA分子是主要遗传物质，人类的各种疾病的感染，多数都可以从相应的DNA分子结构和质量改变上有所体现，因此快速有效称量或检测出DNA的分子质量具有重要的意义。然而，由于DNA分子质量太小，现有的静态质量测量设备无法准确地对其实现测量。因此，设计一种动态测量DNA分子的装置具有重要的意义。

发明内容

[0003] 本发明需解决的技术问题是：针对现有技术存在的技术问题，本发明提供利用一种原理简单、测量精度更高的共振原理测量DNA分子质量的方法。

[0004] 为了解决上述问题，本发明提出的解决方案为：一种利用共振原理测量DNA分子质量的方法，它包括激振器、铰支座A、铰支座B、两端分别装设于所述铰支座A和所述铰支座B上的微简支梁、装设于所述微简支梁上的DNA分子吸附层、待测量的DNA分子、装设于所述微简支梁上的振幅接收器；

[0005] 本发明的利用共振原理测量DNA分子质量的方法由下述步骤组成：

[0006] 采用所述激振器作用所述微简支梁，使其发生自由振动；采用所述振幅接收器接收微简支梁的振幅，并记录下最大振幅与相应的振动频率；确定微简支梁无DNA分子时的固有频率f0；

[0007] 将待测量的DNA分子放置于所述DNA分子吸附层上，采用所述激振器作用所述微简支梁，使其发生自由振动；采用所述振幅接收器接收微简支梁的振幅，并记录下最大振幅与相应的振动频率；采用所述振幅接收器接收微简支梁的振幅，并记录下最大振幅与相应的振动频率；确定有DNA分子时微简支梁的固有频率f1；

[0008] 系统的等效弹簧的等效刚度为k，等效阻尼器的等效阻尼为η，建立DNA分子的共振模型；并利用公式$m=(K/6.28)^2\times(1/f1-1/f0)$计算出DNA分子的质量。

[0009] 本发明与现有技术相比，具有如下优点和有益效果：

[0010] (1)本发明的利用共振原理测量DNA分子质量的方法，通过激振器对微简支梁施加激励，振幅接收器接收微简支梁的最大振幅与相应的频率，从而计算出DNA分子作用前后系统的固有频率改变值。

[0011] (2)本发明的利用共振原理测量DNA分子质量的方法，具有原理简单、测量精度、通过共振原理实现了DNA分子质量的测量。

附图说明

[0012] 图1是本发明的利用共振原理测量DNA分子质量的测量设备结构示意图。

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[0013] 图2是本发明的利用共振原理测量DNA分子质量的力学模型原理图。

[0014] 图中，1—DNA分子；2—机架；21—铰支座A；22—铰支座B；31—DNA吸附层；32—微简支梁；33—等效弹簧；34—等效阻尼器；4—激振器；5—振幅接收器；6—弯曲梁轴线。

具体实施方式

[0015] 以下将结合附图和具体实施例对本发明作进一步详细说明。

[0016] 参见图1所示，本发明的一种利用共振原理测量DNA分子质量的方法，包括激振器4、铰支座A21、铰支座B22、两端分别装设于铰支座A21和铰支座B22上的微简支梁32、装设于微简支梁32上的DNA分子吸附层31、待测量的DNA分子1、装设于微简支梁32上的振幅接收器5。

[0017] 参见图1和图2所示，利用共振原理测量DNA分子质量的方法，由具体步骤如下：

[0018] 参见图1和图2所示，采用激振器4作用微简支梁32，使其发生自由振动；采用振幅接收器5接收微简支梁32的振幅，并记录下最大振幅与相应的振动频率；确定微简支梁32无DNA分子时的固有频率f0；

[0019] 参见图1和图2所示，将待测量的DNA分子1放置于DNA分子吸附层31上，采用激振器4作用微简支梁32，使其发生自由振动；采用振幅接收器5接收微简支梁32的振幅，并记录下最大振幅与相应的振动频率；采用振幅接收器5接收微简支梁32的振幅，并记录下最大振幅与相应的振动频率；确定有DNA分子1时微简支梁32的固有频率f1；

[0020] 参见图1和图2所示，系统的等效弹簧33的等效刚度为k，等效阻尼器34的等效阻尼为η，建立DNA分子1的共振模型；并利用公式$m=(K/6.28)^2\times(1/f1-1/f0)$计算出DNA分子1的质量。

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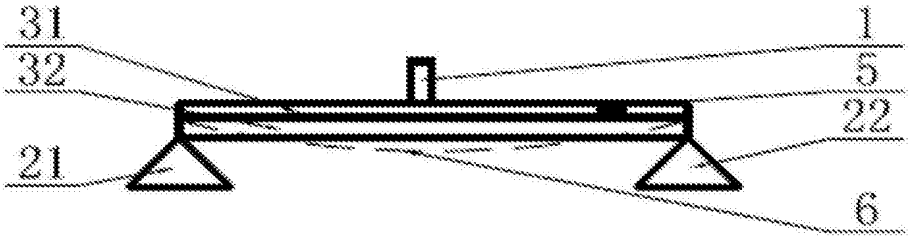


图1

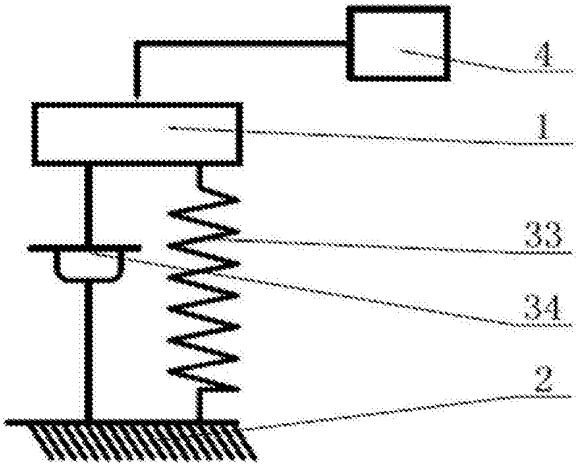


图2

IDT Exhibit 1020.8580

Espacenet

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METHOD FOR THE AMPLIFICATION AND OPTIONAL CHARACTERISATION OF NUCLEIC ACIDS

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Abstract of WO0236821 (A2)

A method for the amplification of a template nucleic acid comprises simultaneously carrying out the steps of reacting a nucleic acid primer with said template nucleic acid, normal DNA precursor nucleotides, at least one modified DNA precursor nucleotide and a DNA polymerase so as to obtain an extended nucleic acid primer, said nucleic acid primer remaining bound to said template; cleaving the modified base-containing extended nucleic acid primer so as to generate a free 3'-OH terminus that is extensible by said DNA polymerase; and repeating steps i) and ii) on DNA fragments thereby generated. The modified precursor nucleotide may be a substrate for a DNA glycosylase or recognised by a 3'-endonulcease and determines the cleavage of the DNA and the site of the cleavage accordingly. The method has significant advantages over existing technologies in that it is more versatile and more flexible with respect to providing a single high throughput process that can be easily adapted to multiple different formats in the fields of DNA detection, quantitation and characterisation.

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(54) Title: METHOD FOR THE AMPLIFICATION AND OPTIONAL CHARACTERISATION OF NUCLEIC ACIDS

(57) Abstract: A method for the amplification of a template nucleic acid comprises simultaneously carrying out the steps of reacting a nucleic acid primer with said template nucleic acid, normal DNA precursor nucleotides, at least one modified DNA precursor nucleotide and a DNA polymerase so as to obtain an extended nucleic acid primer, said nucleic acid primer remaining bound to said template; cleaving the modified base-containing extended nucleic acid primer so as to generate a free 3'-OH terminus that is extensible by said DNA polymerase; and repeating steps i) and ii) on DNA fragments thereby generated. The modified precursor nucleotide may be a substrate for a DNA glycosylase or recognised by a 3'-endonulcease and determines the cleavage of the DNA and the site of the cleavage accordingly. The method has significant advantages over existing technologies in that it is more versatile and more flexible with respect to providing a single high throughput process that can be easily adapted to multiple different formats in the fields of DNA detection, quantitation and characterisation.

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Description

Method for the amplification and optional characterisation of nucleic acids

Technical Field

This invention relates to a new method for amplifying and characterising nucleic acids.

Background Art

Extension of a nucleic acid primer on a target nucleic acid template is a highly important process with multiple applications including nucleic acid detection, diagnosis, and quantitation. In particular, extension of a primer on a template is i) direct evidence that the primer has annealed to the template, ii) confirms the presence of a sequence complementary to the primer on that template, and therefore iii) confirms the presence of that template or target nucleic acid. Using closely related primers that differ by as little as a single base, it is routinely possible to distinguish between nucleic acids that differ in sequence by a single base. The key limitation in using extension of primer on a template nucleic acid as a method for detection, diagnosis, and quantitation of nucleic acids is that in a typical extension reaction, the primer anneals (hybridises) to the template but is only extended once. Thus, the amount of template in a sample determines the amount of primer which is extended. For the majority of applications in the nucleic acid detection, diagnosis, and quantitation fields, the amount of template

is often too small to permit direct detection of the extended primer if that extension reaction is not repeated or cycled in some manner.

Amplification processes are necessary to overcome this key limitation. A variety of these processes have been described previously and they essentially involve either: a) cyclic dissociation conditions where the extended primer is dissociated from the template using heat, thereby allowing annealing and subsequent extension of new primer, b) repeated generation of a primer in DNA through the use of a restriction enzyme to nick an unmodified strand of a hemi-modified DNA recognition site and the ability of a 5'-3' exonuclease deficient DNA polymerase to extend the 3' DNA terminus at the nick and displace the downstream DNA strand, c) where the template nucleic acid is circularised so that primer extension progresses continuously and/or d) where the copies of the template are produced by a transcription method which serve as templates in subsequent primer extension reactions.

Amplification of a nucleic acid (generating multiple copies) to the level where they can be detected and manipulated has a very high utility. Such amplification processes, generating sufficient amounts of a specific target nucleic acid are generally the first key step required in the characterisation of the nucleic acid. Direct amplification of a specific nucleic acid sequence from a sample permits diagnosis of the presence or absence of said nucleic acid in that sample and thus has a very high utility in DNA / gene diagnostics. Direct amplification of a nucleic acid from a sample and subsequent characterisation can be performed for a variety of purposes including diagnosis of the presence or absence of DNA variations such as mutations and polymorphisms in a specific

nucleic acid. Amplification processes can in many cases be designed to permit both amplification and full or partial characterisation of the amplified target.

The current known methods for amplifying and characterising nucleic acids each have different limitations especially with respect to the process required for primer dissociation, primer generation, specificity, versatility, generation of single stranded DNA and facilitation of multiplexing and high throughput genotyping especially genotyping of single nucleotide polymorphisms (SNP) as described below.

Primer extension

Primer extension *per se* on a template is very well documented in the literature. This can be achieved by annealing a primer to its complementary sequence on a template or target nucleic acid followed by incubation with a DNA polymerase in the presence of DNA precursors, typically dATP, dGTP, dCTP and dTTP, which results in extension of the primer from its 3'-OH (hydroxy) terminus in a 5' to 3' direction (with respect to the primer) on the template strand and produces a newly synthesised DNA strand that is complementary to the original template. Primer extension can only occur if the primer has a free 3'-OH terminus. Amplification of this complementary strand from the template by primer extension methods requires that a) primer extension occurs more than once per annealed primer molecule, b) new primer is repeatedly allowed to anneal to the template and be extended

and / or c) that the complementary strand serves as a template for subsequent extension of a second primer.

Polymerase chain reaction (PCR)

Amplification of a nucleic acid template can be achieved by the polymerase chain reaction (PCR) (Saiki, R.K. et al., Science. 239:487-491 (1988).

Typically, amplification of a target segment of a nucleic acid template by PCR is carried out using appropriate synthetic oligonucleotide primers in the PCR along with a thermostable DNA polymerase and DNA precursors. Multiple cycles of denaturation, annealing and primer extension results in the exponential amplification of the target segment. Thus detection of the product indicates the presence of a certain sequence at a specific locus. The length of the amplified product is determined by the combined length of the primers and the distance between their 3' termini on the template and so on.

PCR has an absolute requirement for a thermostable DNA polymerase. It also involves a thermal cycling process, therefore automation of this technique requires specialised equipment. A typical reaction normally requires two primers to initiate the reaction, therefore this adds additional complexity to the reaction, especially when one wants to consider multiplexing several different amplification reactions (i.e. in one tube). This leads to an increased number of primers present in the reaction and thereby increases the possibility of false and nonspecific amplification. In addition, there is also an increased complexity in

designing and optimising the PCR reaction since the annealing temperature of the reaction has to suit both primers used in the reaction. This becomes an additional disadvantage when multiplexing different amplification reactions since a single annealing temperature must suit all amplification reactions being multiplexed.

Also, there is a potential for amplicon contamination since multiple copies of the template nucleic acid are produced during the reaction to act as subsequent templates.

Transcription-based amplification methods

Transcription-based amplification (Kwoh, D.Y. *et al.*, (1989) *Proc. Natl. Acad. Sci. USA, 86* 1173-1177; Guatelli, J.C. *et al.*, (1990) *Proc. Natl. Acad. Sci. USA, 87* 1874-1878; Compton, J. (1991) *Nature 350* 91-92.) is an amplification method that relies on primer extension of primers on a target nucleic acid so that a RNA polymerase promoter is created upstream of the target region to be amplified. Essentially a pair of primers that flank the target sequence are incubated with a template nucleic acid. One of the primers has an RNA polymerase promoter sequence upstream (5') of a sequence that is complementary and anneals to the template. The other primer is complementary to a segment of the complementary template strand. The primer with the RNA promoter sequence is extended on the template strand using a nucleic acid polymerase such as reverse transcriptase and DNA precursors. After thermal denaturation of the hybrid nucleic acid (the template strand and the newly synthesised complementary strand) or enzymatic degradation of the template strand, the second primer anneals to, and is extended on

the newly synthesised complementary strand. This results in a product that is double stranded and has a RNA polymerase promoter attached to the target sequence. Incubation of this product with RNA polymerase and RNA precursors results in production of numerous RNA transcripts of that target sequence. Each RNA transcript serves in turn as a template for the production of a complementary DNA strand and this process continues in a self sustained cyclic fashion under isothermal conditions until components in the reaction become limiting or inactivated. This results in a large amplification of the target nucleic acid sequence.

One main disadvantage is that these techniques have an absolute dependence on RNA which is inherently more unstable than DNA and is more susceptible to degradation. Hence the reaction is ultra sensitive to contaminating ribonucleases. Typically the method requires RNA as template and is not suited or optimal with a DNA template.

Also the method requires at least two primers to initiate the reaction, where one of the primers must be specially designed to incorporate a transcription initiation site for a RNA polymerase.

Furthermore, there is potential for amplicon contamination since multiple copies of the template nucleic acid are produced during the reaction to act as subsequent templates.

These techniques are not suited to mutation or polymorphism detection without the addition of further steps.

Strand Displacement Amplification (SDA)

SDA is an amplification method based upon the ability of a restriction enzyme to nick the unmodified strand of a hemi-modified double stranded DNA at a specific recognition site and the ability of a 5'-3' exonuclease deficient DNA polymerase to extend the 3' terminus at the resulting nick and in doing so, displacing the downstream DNA strand (Walker, G.T. et al., PNAS 89:392-396 (1992)). Exponential target DNA amplification is achieved by coupling reactions on the template in which strands displaced from the reaction on the template strand serve as target for the reaction on the complementary strand and *vice versa*. Essentially heat denaturation of a DNA sample generates two single stranded DNA fragments (T1 and T2). Present in excess are two DNA amplification primers (P1 and P2). The 3' end of P1 binds to the 3' end of T1, forming a duplex with 5' overhangs. Likewise, P2 binds to T2. The 5' overhangs of P1 and P2 contain a recognition sequence for a restriction enzyme such as HincII. An 5'-3' exonuclease deficient form of *E. coli* DNA polymerase extends the 3' ends of the duplexes using the DNA precursors dGTP, dCTP, dTTP and the modified precursor deoxyadenosine 5'-[alpha thio] triphosphate, which produces a hemiphosphorothioate recognition sites on P1T1 and P2T2. HincII nicks the unprotected primer strands of the hemiphosphorothioate recognition sites, leaving intact the modified complementary strands. The DNA polymerase extends the 3' end at the nick on P1T1 and displaces the downstream strand that is functionally equivalent to T2. Likewise, extension at the nick on P2T2 results in displacement of a downstream strand that is functionally equivalent to T1. Nicking and

polymerisation/displacement steps cycle continuously on P1T1 and P2T2 because extension at the nick regenerates a nickable HincII recognition site. Target amplification is exponential because strands displaced from P1T1 serve as targets for P2 and strands displaced from P2T2 serves as targets for P1.

A major disadvantage of SDA is that one must use specially designed primers which incorporate a site for a specific restriction enzyme. Typically one requires two or more sequence specific primers *per* amplicon to carry out the reaction. In addition, the amplified fragments produced in this reaction using a single primer do not readily have a defined 3' terminus. This is also a major disadvantage of SDA since a defined 3' terminus on a displaced fragment allows one to prime a subsequent reaction with this same fragment.

WO 97/03210 discloses a method for rapidly detecting the presence or absence of a particular nucleic acid sequence at a candidate locus in a target nucleic acid sample comprising the steps of: 1) introducing a modified base which is a substrate for a DNA glycosylase into said candidate locus at one or more preselected positions; ii) excising the modified base by means of said DNA glycosylase so as to generate an abasic site; iii) cleaving phosphate linkages at abasic sites generated in step ii); and iv) analysing the cleavage products of step iii) so as to identify in said target nucleic acid sequence the presence or absence of said particular nucleic acid sequence at said candidate locus. The method has particular application for detecting specific mutations in a DNA sample, including the detection of multiple known mutations in DNA.

WO 99/54501 discloses a method for characterising nucleic acid molecules comprising the steps of i) introducing, a modified base, for example uracil, which is a substrate for a DNA glycosylase into a DNA molecule; ii) excising the modified base by means of said DNA glycosylase so as to generate an abasic site; iii) cleaving the DNA at the abasic site so as to generate an upstream DNA fragment that can be extended; and iv) incubating the extendible upstream fragment in the presence of an enzyme, for example, a polymerase or a ligase, allowing for extension thereof and a template nucleic acid and analysing the resultant fragment(s). However, in the case of the method described in WO 99/54501 for the cleavage of the DNA at the site where the base was excised is critical.

In the case of the method as exemplifed in WO 99/54501 the reaction mixture bearing the amplified target nucleic acid was treated with exonuclease I to digest the primers not extended in the amplification step and shrimp alkaline phosphatase to digest dNTPs not incorporated during the amplification step. Accordingly, no further amplification of the template nucleic acid occurred and the method was limited to a single cycle.

Consequently, it is important to develop an improved method for amplification and characterisation of nucleic acids that is more versatile, more specific, offers higher throughput, facilitates multiplexing of reaction and permits generation of single stranded DNA.

Accordingly, the invention provides a method for the amplification of a template nucleic acid, which comprises simultaneously carrying out the steps of:

i) reacting a nucleic acid primer with said template nucleic acid, normal DNA precursor nucleotides, at least one modified DNA precursor nucleotide and a DNA polymerase so as to obtain an extended nucleic acid primer, said nucleic acid primer remaining bound to said template;

ii) cleaving the modified base-containing extended nucleic acid primer so as to generate a free 3'-OH terminus that is extendible by said DNA polymerase; and

iii) repeating steps i) and ii) on DNA fragments thereby generated.

In one embodiment, the modified base-containing extended nucleic acid primer is cleaved by a 3'-endonuclease.

In this embodiment, preferably, the 3'-endonuclease is Endonuclease V from *E. coli* or homologues thereof to be found in other organisms.

In an alternative embodiment, the method comprises the steps of:

i) reacting a nucleic acid primer with said template nucleic acid, normal DNA precursor nucleotides, at least one modified DNA precursor nucleotide which is a substrate for

a DNA glycosylase, and a DNA polymerase so as to obtain an extended nucleic acid primer, said nucleic acid primer remaining bound to said template;

ii) excising the modified base of the modified DNA precursor nucleotide from the extended nucleic acid primer by means of a DNA glycosylase so as to generate an abasic site;

iii) cleaving the extended nucleic acid primer at the abasic site so as to generate a free 3'-OH terminus that is extendible by said DNA polymerase; and

iv) repeating steps i)-iii) on DNA fragments thereby generated.

The method according to the invention has many specific advantages as set out below. However, more generally the method according to the invention has significant advantages over existing technologies in that it is more versatile and more flexible with respect to providing a single high throughput process that can be easily adapted to multiple different formats in the fields of DNA detection, quantitation and characterisation.

The invention will be described principally hereinafter with reference to the embodiment involving the use of a DNA glycosylase. We have coined the term glycosylase mediated amplification (GMA) for this method according to the invention. However, all embodiments of the invention are referred to collectively herein under the acronym GMA.

In the method according to the invention the modified DNA precursor nucleotide can thus be a substrate for a DNA glycosylase or recognised by a 3'-endonuclease as described herein.

Typically, the nucleic acid template strand can be any strand from a natural or artificially synthesised nucleic acid.

Preferably, the template nucleic acid is DNA.

The method according to the invention is primed/initiated by the nucleic acid primer. For convenience, the primer responsible for initiating the reaction is referred to herein as the initiating primer (IP).

The IP can be any nucleic acid with a free 3'OH terminus that can be extended by a DNA polymerase. The IP may be artificially synthesised for example a synthetic oligonucleotide, or derived directly or indirectly from a naturally occurring nucleic acid.

In one embodiment, the nucleic acid primer is a DNA primer.

The normal DNA precursor nucleotides are the deoxyribonucleotide triphosphates dATP, dCTP, dGTP and dTTP. In certain limited circumstances, dideoxynucleotide triphosphates may also be used and included in the reaction. Therefore, possible normal precursors also include ddATP, ddCTP, ddGTP and ddTTP.

Preferably, the DNA precursor nucleotides are selected from dATP, dCTP, dGTP and dTTP.

Any one of several nucleic acid polymerases may be used in GMA. When a DNA template is used a DNA polymerase is used. When an RNA template is used a DNA polymerase which can utilise a RNA template is required, typically such an enzyme is reverse transcriptase. Typically, two classes of DNA polymerases are used depending on whether displacement or digestion of the nucleic acid downstream of the extending primer is required. When strand displacement is required, thereby generating displaced fragments, a DNA polymerase that does not have a 5'-3' exonuclease activity is used. In contrast, when a DNA polymerase with 5'-3' exonuclease activity is used, the downstream DNA is degraded in each cycle of the GMA reaction. This also leads to generation of a detectable product as discussed further below.

There are several known modified precursor nucleotides which, when incorporated into DNA, become a substrate for a DNA-glycosylase and/or are recognised by a 3'-endonuclease, as appropriate. In the latter case, the modified precursor nucleotide directs cleavage by a 3'-endonuclease enzyme to a phosphodiester bond 3' to the site of incorporation thereof.

In each case cleavage is dependent on the presence of the modified base in the DNA and it is this that dictates the cleavage of the DNA and dictates the location of the cleavage, in either of two ways, namely, 1) excision of the modified base by the glycosylase and cleavage of the subsequent abasic site or 2) cleavage of the extended nucleic acid primer at the second phosphodiester bond on the 3' side of the site of incorporation of the modified base by a 3'-endonuclease

enzyme. In one embodiment, the modified nucleic acid precursor is dUTP.

The modified precursor nucleotide dUTP is a base sugar phosphate comprising the base uracil and a sugar phosphate moiety. Primer extension on template using the precursor nucleotides dATP, dCTP, dGTP, and dUTP in place of dTTP, results in newly synthesised DNA complementary to the template where thymine is replaced completely by uracil.

However, it will be appreciated by those skilled in the art that other modified nucleic acid precursors can also be used, such as dITP and 8-OH dGTP.

The modified precursor nucleotide dITP is a base sugar phosphate comprising the base hypoxanthine and a sugar phosphate moiety. The modified precursor nucleotide 8-OH dGTP is a base sugar phosphate comprising the base 8-OH guanine and a sugar phosphate moiety.

The glycosylase substrate precursors dUTP, dITP and 8-OH dGTP when incorporated into DNA generate the glycosylase substrate bases uracil, hypoxanthine and 8-OH guanine, respectively.

In one embodiment, the DNA glycosylase is uracil DNA-glycosylase (UDG).

Uracil in DNA is recognised specifically by UDG and released from DNA. UDG also recognises other uracil related bases when present in DNA.

Many DNA-Glycosylases have been described. These enzymes cleave the N-glycosidic bond connecting the glycosylase substrate base to the DNA backbone. This releases the base from the DNA and generates an abasic site.

Other suitable DNA glycosylases include alkylpurine DNA-glycosylases (ADG) or formamidopyrimidine DNA-glycosylase (FPG) DNA-glycosylase.

Hypoxanthine is recognised specifically by alkylpurine DNA-glycosylases (ADG) and released from DNA. This enzyme also recognises and releases N3 methyladenine, N3 methylguanine, O^2 methylcytosine and O^2 methylthymine when present in DNA. 8-OH guanine is recognised specifically by FPG DNA-glycosylases and released from DNA. This enzyme also recognises and releases ring opened purines when present in DNA.

Several agents are known which cleave the phosphodiester bonds in nucleic acids at abasic sites. Cleavage of the bond can be 5' of the abasic site or 3' of the site. 5' cleavage can occur proximal or distal to the phosphate moiety and generate an upstream fragment which has a 3' terminus with a free 3'OH group or 3'-phosphate group respectively. 3'-OH termini are extendible by DNA polymerases on a template whereas 3'-phosphate termini are not extendible. Such 3'-phosphate termini can generally be rendered extendible by treatment with a phosphatase enzyme such as T4 polynucleotide kinase which has a 3' phosphatase activity. Agents which cleave 5' to the phosphate moiety and generate a 3' terminus with a free 3'OH are the enzymes with AP endonuclease

activity, such as AP endonuclease IV from *E. coli.* Agents which cleave 3' to the phosphate moiety and generate a 3' terminus with a 3'-P group are alkali, heat and certain DNA repair enzymes such as FPG and basic proteins and peptides. Agents which cleave 3' of the abasic site include heat, and DNA repair enzymes with AP lyase activity such as endonuclease III from *E. coli.* Such 3'-deoxyribophosphate (dRp) termini can generally be rendered extendible by treatment with an AP endonuclease. FPG-DNA glycosylase cleaves both 5' and 3' of the abasic site.

Preferably, the extended nucleic acid is cleaved at the abasic site by means of an enzyme which cleaves at a nucleic acid abasic site.

Further, preferably, the enzyme is an AP endonuclease, especially AP endonuclease IV which cleaves 5'of the abasic site and generates a free 3'OH terminus.

Where the extended primer is cleaved by a 3'-endonuclease, cleavage of the extended primer by such an enzyme is dependent on the presence of a modified base in the extended primer and cleavage occurs at a phosphodiester bond on the 3' side (i.e. downstream) of the site of incorporation of the modified base. In contrast to the action of the glycosylase and AP site cleavage, cleavage of the extended primer by the 3'-endonuclease does not involve excision of the modified base and does not involve creation of an abasic site. Cleavage by the 3'-endonuclease is dependent on the presence of a modified base in the extended primer and recognition of said modified base by the enzyme. Cleavage usually occurs at the second phosphodiester bond on the 3' side of the modified

base/nucleotide. This cleavage event results in the generation of a nick in the DNA strand with a 3'-OH and a 5'-phosphoryl group being generated. The DNA polymerase present in the reaction can then extend from the free 3'OH group. As mentioned above, the 3'endonuclease may be Endonuclease V from *Escherichia coli*. Endonuclease V recognises several modified bases in DNA including uracil, hypoxanthine (inosine) and urea residues. In addition to cleavage of DNA with modified bases, endonuclease V can also cleave DNA containing abasic sites. Therefore in certain circumstances, endonuclease V could be used to cleave the extended primer in combination with the action of a DNA glycosylase which generates an abasic site in the DNA.

In one embodiment, the modified precursor nucleotide partially replaces one of the normal precursor nucleotides.

For example, primer extension on a template using the precursor nucleotides dATP, dCTP, dGTP and dTTP in addition to the modified precursor nucleotide dUTP results in newly synthesised DNA complementary to the template where thymine is replaced randomly by uracil. The uracil is incorporated in the newly synthesised DNA strand at positions complementary to adenine residues in the template DNA strand during the DNA synthesis process. Thus, the downstream displaced fragments are delimited by the position of the random incorporation of the dUMP in the newly synthesised DNA. This results in the generation of displaced fragments of multiple sizes according to the permutations of dUTP *versus* dTTP incorporation in the complementary strand opposite A residues in the template nucleic acid strand.

A similar approach substituting dITP for dGTP or 8-OH dGTP for dGTP leads to replacement of all or part of dGTP in the newly synthesised DNA complementary to the template by hypoxanthine or 8-OH guanine, respectively at positions complementary to cytosine residues in the template DNA strand. Replacement of all or part of one or more of the regular DNA precursors with one or more dideoxy terminator nucleotides (a nucleotide that prohibits further extension of a primer on a template once incorporated) can be used to terminate primer extension by a DNA polymerase when desired. Replacement of part of one or more of the regular DNA precursors with one or more dideoxy terminator nucleotides terminates primer extension at multiple different positions on the template strand and generates terminated primers of multiple different lengths. Replacement of all of one or more of the regular DNA precursors with one or more dideoxy terminator nucleotides terminates primer extension at specific positions on the template strand and generates terminated primers of specific lengths. More than one modified precursor nucleotide may be used in the GMA with one or more DNA-glycosylases. Two DNA glycosylases may be used whereby one releases a modified base from the primer while the other releases the modified base once incorporated into newly synthesised DNA.

Steps i) and ii) or i)-iii), as appropriate of the method according to the invention can continue in a cyclical manner until one of the reagents becomes limiting.

The method according to the invention can be carried out under isothermal conditions.

Accordingly, when the method is isothermal, no thermocycling is required.

The method according to the invention is the only isothermal amplification reaction capable of amplifying multiple, newly synthesised and discrete DNA segments in a primer extension reaction using a single primer.

It will be appreciated that the method according to the invention can result in the accumulation of displaced single stranded downstream fragments of nucleic acid specified by the locations of modified bases in a complementary nucleic acid strand.

Thus, the method according to the invention provides a means of generating multiple copies of discrete single stranded primers downstream of an initiating primer. This offers exceptional specificity for detection purposes, as the discrete downstream primers can only be generated if the target template nucleic acid is present. Thus for DNA diagnosis, GMA is a significant improvement over any previously described amplification method with respect to specificity.

Amplification of multiple DNA segments from a single template sample is highly desirable and is currently a limitation for amplification technologies. This limitation largely arises from the fact that existing technologies use exponential amplification and/or are more cumbersome and generate double stranded product or large size single stranded product.

The method according to the invention offers significant advantages over existing methods for multiplex amplification of DNA segments.

The method according to the invention can be used for generating multiple copies of discrete single stranded primers downstream of an initiating nucleic acid primer.

The displaced downstream fragments can be extended in a secondary reaction.

Furthermore, the displaced downstream fragments can be extended on a secondary template nucleic acid.

Also multiple secondary templates can be immobilised on a DNA chip.

These aspects of the invention will be described further below.

The method according to the invention can be used in detection diagnostics. Thus, for example the method can be used in the detection of pathogens.

The method according to the invention can also be used in the detection of the presence or absence of mutations and in the detection of polymorphisms.

It will be appreciated that the method according to the invention can be used in the quantification of the level of a nucleic acid in a sample.

The GMA method according to the invention can be measured both qualitatively and quantitatively by several means. Therefore, the characteristics and quantity of an IP and / or its complementary annealing site on a template nucleic acid can be assessed by the ability of the IP to prime a GMA reaction on that template. The resolution achieved according to the invention, if desired, can be as high as determining single base differences between IPs and /or the template or target nucleic acid and this is based on the successful priming of a GMA reaction. Since the IP may be derived directly or indirectly from a naturally occurring nucleic acid, and the GMA permits qualitative and quantitative characterisation of the IP, the GMA can therefore be used to qualitatively and quantitatively characterise nucleic acids. This has high utility in the fields of detection, diagnosis, and quantitation of nucleic acids. This includes for example, detection and quantitation of pathogenic micro-organisms like certain bacteria and viruses, detection of variants therein, detection of human disease causing mutations, detection of single nucleotide polymorphisms and quantification of the amount/titre of specific mRNA species in tissue samples.

The method according to the invention has significant advantages over existing technologies in the area of quantitation. As the kinetics of the GMA are linear, the GMA reaction is easier to detect and

measure/quantify than existing amplification technologies with exponential kinetics.

The method according to the invention also has significant advantages over existing technologies in the area of contamination control. The reason for this is that unlike existing technologies, GMA in its basic format does not synthesise new templates for subsequent use by initiating primers and the kinetics of the process are linear.

The method according to the invention can also be used in signal amplification from any nucleic acid that can function as a primer or template.

The method according to the invention has significant advantages over existing technologies in that it permits signal amplification from an initiating primer using a single linear template. The GMA does not incorporate the initiating primer into the amplified displaced downstream fragments. This offers the advantage that the displaced downstream fragments do not have a 5' tail that is always the initiating primer. Furthermore, this means that the displaced downstream fragments can be extended in a secondary reaction to produce complementary displaced downstream fragments devoid of sequences complementary to the initiating primer.

The method according to the invention is unique in that it can be carried out in a single reaction vessel whereby extension of an IP on a template generates and amplifies new primers that are distinct from the

IP and which can subsequently serve as IPs on the same template from which it was derived or on a different template.

Brief Description of Drawings

Fig. 1 is a flow diagram of one embodiment of the method according to the invention as described *inter alia* in Example 1; and

Fig. 2 is a flow diagram of another embodiment of the method according to the invention as described in Example 4.

One embodiment of the invention is illustrated with reference to Fig. 1. as follows:

Event 1 The primer binds to a complementary sequence on the template;

Event 2 Once bound the free 3'OH terminus of the primer is extended by the DNA polymerase. This happens through the polymerisation of the precursor nucleotides onto the 3'OH terminus of the primer. The precursor nucleotides (dATP, dCTP, dGTP and / or dTTP) are incorporated into the extending primer in addition to the modified precursor nucleotide according to the sequence of the template. The modified precursor nucleotide usually replaces either fully or partially one of the normal precursor nucleotides. The newly synthesised DNA strand is complementary to the initial template and is referred to as the complementary template strand herein;

Event 3 Once the modified precursor nucleotide is incorporated into the newly synthesised DNA, that DNA then contains a modified base which is a substrate for the specific DNA glycosylase. Consequently every time a modified base appears in the newly synthesised DNA, it is released from the DNA by cleavage of the N-glycosylase bond that joins that base to the deoxyribose moiety in the DNA. This results in the production of an abasic site which is essentially a deoxyribose moiety joined to the flanking DNA by a phosphodiester bond on the proximal and distal side (i.e. 5' and 3' of the deoxyribose moiety with the 5' bond being the closest to the original primer); and

Event 4 The abasic site is for example a substrate for AP endonuclease (APE) enzyme. Consequently every time an abasic site appears, it is cleaved by APE. This enzyme cleaves the phosphodiester bond 5' of the deoxyribose moiety generating a free 3'OH terminus on the upstream DNA segment and a deoxyribose moiety attached to the 5' end of the downstream segment.

Event 5 The DNA polymerase present in the reaction synthesises new DNA from this newly generated 3'OH terminus of the upstream fragment every time it is created, and in doing so, displaces the DNA downstream of the polymerisation as a single strand. This results in the incorporation of new precursors nucleotides, including modified precursor nucleotides, into the newly synthesised complementary

template strand and the appearance of a new modified base at each position in the newly synthesised strand opposite its complementary base in the template nucleic acid.

Thus, the reaction steps i) to iii) according to this embodiment cycle continuously until one of the reagents becomes limiting.

Each free 3'OH terminus created in a cycle of the reaction is extended once in each subsequent cycle of the reaction with concomitant displacement of the downstream DNA segments. As the reaction is continuous the net result is the repeated synthesis of new DNA from each 3'OH terminus created and accumulation of the displaced downstream DNA as discrete single stranded fragments of discrete sizes, referred to herein as displaced downstream fragments, or displaced fragments, delimited by the locations of modified bases in the complementary strand and/or the 3' terminus due to termination of DNA synthesis by the polymerase.

Primers that are extended and cleaved can be immediately re-extended by the polymerase by incorporation of nucleotides including modified precursor nucleotides. Since the normal precursor nucleotides, modified precursor nucleotides, polymerase, glycosylase and cleavage agents are all simultaneously present in the same reaction, a continuous cycle of extension and cleavage results in the amplification of multiple copies of displaced downstream fragments.

When the modified precursor nucleotide is dUTP, then the modified base is uracil and the specific DNA glycosylase when such is

used is Uracil DNA glycosylase. Hence the displaced downstream fragments are delimited or defined by the locations of uracil in the complementary strand and therefore by the location of adenine bases in the template nucleic acid, since uracil forms a normal Watson-Crick base pair with adenine.

Therefore, briefly, the primer binds to the template and is extended by the DNA polymerase. DeoxyATP, dCTP, dGTP, and dUTP, are incorporated into the extending primer. Uracil DNA glycosylase then excises the uracil bases in the newly synthesised strand and the resulting abasic sites are cleaved by the AP endonuclease enzyme.

Alternatively, the 3'-endonuclease recognises uracil in the newly synthesised strand and cleaves the strand at the second phosphodiester 3' of the uracil moiety.

The DNA polymerase then begins to synthesise new DNA from the newly generated 3'OH termini every time they are created and displaces the 3' or downstream DNA as the polymerisation proceeds. This again results in the incorporation of more uracil into the newly synthesised DNA which is subsequently excised and/or recognised `, the DNA cleaved and polymerisation initiated from the new 3'OH terminus.

The GMA may be carried out at mesophilic or thermophilic temperatures. A DNA polymerase such as the *E. coli* DNA polymerase Klenow fragment exo$^-$ may be used at mesophilic temperatures (usually between 25°C and 42°C and typically at 37°C) while at thermophilic

temperatures (typically between 50°C and 80°C, although it can be higher), thermostable DNA polymerases such as a strand displacing DNA polymerase from *Thermus aquaticus* (Stoffel fragment) may be used. Both types of polymerase may be added jointly or sequentially to a reaction. When high processivity is required, so that a primer is extended to a considerable length before the polymerase disassociates from the DNA, a highly processive polymerase can be used. By contrast, when low processivity is required, so that a primer is extended to a short length before the polymerase disassociates from the DNA, a lowly processive polymerase can be used. When an RNA template is used, a reverse transcriptase with strand displacement activity may be used for the GMA reaction.

In the simplest case, an artificial or synthetic primer is supplied as the IP to prime the GMA reaction on a given template or target nucleic acid. The IP is chosen so that it hybridises to a specific target sequence in the template. Following hybridisation of the IP, the GMA initiates and the extended IP is repeatedly extended in the cyclic reaction resulting in amplification of the displaced downstream DNA fragments. These displaced fragments can be qualitatively and quantitatively characterised by multiple different approaches according to published procedures.

Direct detection of displaced fragments can be achieved by a variety of means, for example they may be suitably labelled.

Labelling of the displaced fragments can be performed by a variety of means including addition of a radioactive, fluorescent, or detectable ligand to the fragments during or post synthesis. The use of a

labelled precursor nucleotide in any of the extension reactions facilitates detection of these fragments. Direct DNA staining methods such as silver or ethidium bromide staining facilitates their detection after size separation based on electrophoretic mobility. Hybridisation of complementary or test nucleic acids to these fragments may be used to identify them and such complementary or test nucleic acids may be immobilised and hybridised directly to the displaced fragments. In this context, DNA macroarrays, DNA microarrays and DNA chips are very suitable. Alternatively, the displaced fragments may serve as a bridging hybridisation molecule so that one test nucleic acid, which may be immobilised, is hybridised to part of a displaced fragment and a second test or reporter nucleic acid hybridises to the remainder of the displaced fragment. Again, DNA macroarrays and DNA microarrays are also very suitable in this context.

The complementary or test nucleic acids may suitably be labelled by any of a variety of direct or indirect labelling approaches such as reporter-quencher fluorescent dye methods. Since the displaced fragments are single stranded, hybridisation to complementary molecules will create double stranded nucleic acids which may be detected using double stranded specific probes such as SYBR green. This may be achieved in the GMA reaction according to the invention by including DNA complementary to the displaced fragments in addition to SYBR green reagent which specifically binds double stranded DNA.

The sequence of the displaced fragments and, in particular, the 3'end thereof can be determined by their ability to function as initiating primers in a subsequent or the same GMA reaction. Essentially, such

determination is based on the ability of these fragments and particularly their 3'end to hybridise to a selected complementary sequence on a template under selected conditions and to function as an IP in a secondary GMA reaction. It will be appreciated that multiple possibilities exist for the selection of complementary sequences on a template and template molecules themselves. Nonetheless, the ability of a displaced fragment to function as an IP in its own right in a GMA reaction is a measure of its hybridisation to, or lack of hybridisation to, a selected target sequence and thus the determination of the nature of the sequence of part or all of the displaced fragment is on this basis.

Detection of the displaced fragment is highly advantageous from a specificity perspective since the generation thereof is dependent on a) the successful hybridisation of the IP to the target template and b) priming of a GMA reaction on the correct template. Thus, detection of the anticipated displaced fragment is evidence that the IP has hybridised to the correct region on the correct template.

The identity or sequence of the displaced fragment can be determined using a variety of approaches including hybridisation, mass measurement, and the ability thereof to be ligated directly to a nucleic acid or its ability to act as the complementary strand necessary for ligation of one or more nucleic acid molecules. For example, it can be detected and characterised by assessing the ability thereof to serve as a bridging hybridisation molecule for ligation of the 5' and 3' end of a linear test DNA to form a circle. The hybridised displaced fragment, or an additional primer, can then serve as an IP for a GMA reaction on the

new circular template resulting in amplification of the DNA by a rolling circle replication (RCR) mechanism.

It should also be noted that, in addition to acting as an IP, a displaced downstream fragment can also act as a template in a subsequent GMA reaction.

There are many methods by which an IP can be generated. In all cases the IP supplied or generated must have a free 3'OH terminus so that it can prime the subsequent DNA polymerisation step.

Artificial synthesis of an IP allows for numerous possibilities with respect to synthesis, design and modification of the IP. Many different modifications of artificially synthesised primers have been previously described. These include modifications of the base, sugar and phosphodiester bond and including those whereby glycosylase substrate bases such as uracil, hypoxanthine and 8-hydroxy guanine are incorporated into the primer.

Typically a standard or modified IP is synthesised to specifically match all or part of its complementary sequence on the template nucleic acid. It is well established that complementarity between the bases at the 3' end of a potentially extendable primer and the template is one of the key parameters that determines whether the IP will be extended on that template. An IP that is fully complementary to a section of the template can be extended by DNA polymerase, whereas an IP that is fully complementary to a section of the template except for the base at its 3' terminus is not extended under stringent conditions. Thus, it will be

appreciated that extension of an IP on a template can be used to differentiate between closely related IPs that differ by as little as a single base. Similarly, it will be appreciated that extension of an IP on a template can be used to differentiate between closely related templates that differ by as little as a single base. This approach is of particular importance in the field of human genetics where it permits detection of mutations and polymorphisms such as single nucleotide polymorphisms (SNPs).

It is well established that the stringency of hybridisation conditions between IP and a template can be varied considerably. Low stringency conditions permit low specificity hybridisation between DNA molecules. Thus under low stringency conditions, DNA molecules that are partially complementary can hybridise to each other. Therefore, under such conditions a partially complementary IP could hybridise to a template. Under such conditions, a single IP can hybridise and be extended at one or more partially complementary sites on a template nucleic acid. When the stringency conditions are so low that priming occurs at multiple sites, the process is referred to as random priming (although the priming is not entirely random in that a significant match between the five most 3' bases of the IP and the template is usually required). As stringency is increased, hybridisation becomes increasingly specific and hybridisation conditions can readily be found that permit only hybridisation of a fully complementary primer, but excludes hybridisation of a partially complementary primer even if it only differs by a single base. During hybridisation, there are a number of parameters by which the stringency of hybridisation may be altered, such as

temperature. As temperature is increased, the stringency of hybridisation increases. Consequently, in enzymatic processes that are dependent on hybridisation between DNA molecules, higher specificity can be achieved at higher temperatures. However, the enzymatic process at the higher temperature usually requires thermostable enzymes.

The IP may be generated following cleavage of an artificially synthesised primer or of a natural nucleic acid such that a new free 3'OH terminus is produced. The cleavage can be either single or double strand dependent, dependent on the presence of a modified base in a single strand or double strand context, sequence dependent, dependent on the presence of a mismatch, or dependent on the presence of a specific structure. Thus, a primer may be generated through glycosylase mediated cleavage of a probe bearing a modified base that is recognised by a specific glycosylase in a single strand or double strand context. An IP may also be generated by cleavage of a probe bearing a mismatched base that is recognised by a mismatch specific endonuclease or glycosylase in a double strand context, for example when the probe is annealed to the template nucleic acid. This can be achieved by designing the primer so that a base mismatch is created between the primer and the template at one or more locations in the hybridised segment and incubating with one or more of a variety of endonucleases or DNA glycosylases which have been shown previously to specifically cleave at mismatched bases in double stranded nucleic acids. These include T7 endonuclease I, MutY DNA glycosylase, thymine mismatch DNA glycosylase and endonuclease V.

An IP may also be generated through cleavage of a complex formed by the hybridisation of overlapping oligonucleotide probes using a structure-specific enzyme such as a cleavase.

A primer may be synthesised with a blocked 3' terminus so that extension of the primer is not possible unless the blocking group is released through cleavage of the primer. Such a primer is referred to as a 3'-blocked primer herein. A blocked 3' terminus of a primer can be achieved by several methods including 3' phosphorylation, incorporating a dideoxy nucleotide at the 3' terminus, synthesising the primer with a 3'amine or 3'thiol group, synthesising the primer with one or more inverted nucleotides at the 3' terminus, or cleaving an abasic site with a DNA lyase.

Therefore in one embodiment of the invention, a primer may be synthesised with a noncomplementary 3' terminus (i.e. not complementary to the template at its 3' terminus) so that extension of the primer is not possible unless the 3' terminus is released through cleavage of the primer.

A primer may be synthesised with a noncomplementary 5' terminus so that cleavage causes dissociation of the primer from template nucleic acid. The dissociated primer can then serve as an IP in a GMA reaction on a different template.

Cleavage of a primer so that a 3'-blocked primer is unblocked, and a noncomplementary 3' terminus or noncomplementary 5' terminus is released, may be made dependent on hybridisation of the primer to all or

part of a template nucleic acid so that following the cleavage, an IP with a new free 3'OH terminus is created, permitting extension of the IP on the same or a distinct template. Cleavage of the hybridised primer in this instance so that it can extend on a template, requires that the primer is cleaved at one or more locations in the hybridised segment of that primer. This can be achieved by designing a primer containing the modified base hypoxanthine which is a substrate for 3-alkylpurine DNA glycosylase (e.g. AlkA).

In a further embodiment of the invention, using thermostable cleaving agents, it is possible to cleave a hybridised primer at a modified or mismatched base so that once the probe is cleaved, the two or more fragments become thermally unstable and fall off the target nucleic acid, thereby allowing another full-length primer to hybridise. This oscillating process amplifies the signal (increased generation of the cleaved primer). The cleaved product with a 3'OH terminus can then serve as an IP in a subsequent or coupled GMA reaction.

An IP may also be generated by cleavage of a primer at a modified base where such cleavage is dependent on extension of the primer on a template. This permits generation of an IP smaller than the original primer and which can be characterised in a variety of ways. For example, *E. coli* Uracil DNA Glycosylase will not release uracil from the ultimate or penultimate 3' position of a primer. However, if the primer is extended on a template, the uracil which was at the ultimate or penultimate 3' position of the primer is now further away from the 3' terminus of the newly extended nucleic acid and will therefore be released. Thus a primer with a free 3'OH terminus and one or two bases

shorter than the original primer is generated and is therefore an IP for a subsequent GMA reaction.

An IP may be generated from a naturally occurring or amplified nucleic acid by full or partial enzymatic or chemical cleavage with DNA or RNA cleaving agents such as DNAses, RNAses, restriction endonucleases, DNA glycosylases following conversion of a normal DNA base to a glycosylase substrate base or incorporation of such a base during amplification, AP endonucleases following partial or full depurination or depyrimidination of DNA, enzymes that cleave RNA or DNA in RNA:DNA hybrids such as RNAseH and enzymes that cleave at DNA mismatches formed following denaturing and re-annealing of nucleic acid hybrid molecules such as RNA:DNA hybrids and DNA:DNA hybrids. Enzymatic or chemical cleavage of DNA or RNA may generate 3' termini that are not extendible by DNA polymerases. Such 3' termini can generally be rendered extendible by treatment with one or more enzymes such as AP endonuclease IV or T4 polynucleotide kinase which has a 3' phosphatase activity. It is well established that cleavage agents can be double strand, single strand or sequence specific. A double or single stranded nucleic acid may be cleaved to small double or single stranded fragments, respectively using one or more cleavage agents prior to GMA. This provides a means to restrict the size of the displaced fragments.

Cleavage or nicking of one strand of a duplex molecule provides a section of nucleic acid with a free 3'OH terminus that can function directly as an IP on the template to which it is hybridised or on a distinct

template in a GMA reaction. Cleavage or nicking of one strand of a duplex molecule can be achieved using certain cleavage agents. The nucleic acid can be nicked non-specifically with a low amount of nuclease enzyme such as DNAse, leading to the generation of multiple different displaced downstream fragments from multiple locations on the nucleic acid template.

The nucleic acid can be nicked with more specific agents such as the restriction enzyme N.BstNB I which nicks DNA four bases downstream of the 3' side of the recognition sequence GAGTC. Alternatively, the nucleic acid can be nicked with a restriction enzyme, for example HincII or BsoBI, which nicks the unmodified strand of a hemi-modified double stranded DNA at a specific recognition site, thereby generating a free 3'OH terminus. RNA may be cleaved non-specifically with certain RNAses and more specifically with sequence or structure specific RNAses such as ribozymes. RNAseH cleaves RNA in a RNA:DNA hybrid. Thus RNA can be cleaved by RNAseH following synthesis of a cDNA on the RNA template by reverse transcriptase. The RNA can be cleaved specifically by RNAseH following annealing of oligonucleotide DNA molecules to one or more sequences on the RNA.

In particular, addition of oligo / poly dT provides a means to make the 3'polyA tail of mRNA species double stranded. Addition of RNAseH results in digestion of the polyA tail providing a unique 3' terminus for each mRNA species. This method provides a means whereby each mRNA species in a sample can potentially act as an IP in a GMA.

A double stranded nucleic acid may be cleaved to expose its free 3'OH terminus enabling the cleaved DNA to function as an IP in a GMA. For example, a double stranded DNA may be denatured by heat. Alternately, it may be treated with the T7 gene 6 exonuclease which hydrolyses duplex DNA in a stepwise non-processive reaction from the 5' termini. The enzyme is not active on single stranded DNA and thus stops when duplex regions are no longer present.

Importantly, specific cleavage of one strand of a duplex molecule at a mismatch generates a nucleic acid fragment with a 3'OH terminus that can function directly as an IP on that template to which it is hybridised or on a different template. This provides a means of identifying mutations and polymorphisms in nucleic acids.

In this respect, the present invention allows one to investigate the presence or absence of a mutation or polymorphism at a specific location in a nucleic acid (candidate locus) in the following way: annealing a 3'-blocked primer to the target nucleic acid such that a mismatch is generated at the candidate locus. The mismatch is typically located internally with respect to the primer, i.e. that the base creating the mismatch with the candidate locus base on the template is not the base residue of the primer's 5' terminal or 3' terminal nucleotide. Upon cleavage of the primer at the position of the mismatch with a mismatch specific glycosylase and abasic site cleavage agent, the 5' cleaved section of the primer has a 3'OH terminus and is therefore an IP which can now initiate a GMA reaction on that same template or an alternative template. Therefore, the resultant GMA is indicative of the presence or

absence of the mutation dependent on whether a mismatch was formed or not by the binding of the primer to the template and *vice versa*.

Additionally, reannealed nucleic acid hybrid molecules can be treated with mismatch specific enzymes such as T7 endonuclease I, MutY DNA glycosylase, thymine mismatch DNA glycosylase or endonuclease V. Combinations of gene amplification by PCR methods followed by reannealing and cleavage of DNA at mismatches with a mismatch specific repair enzyme generates free 3'OH terminii that can serve as IPs in a subsequent GMA reaction on a distinct template following dissociation from its complementary strand.

Alternatively, extension from the 3'OH terminus generated at the mismatched site using a strand displacement DNA polymerase in a standard "once off" strand displacement reaction, or in a GMA reaction, produces a displaced fragment that can serve as an IP in a subsequent GMA reaction. Since the generation of the IP is dependent on the presence of a mismatch, priming of a GMA reaction is indicative of a mismatch. When a whole genome amplification method is used with this approach with multiple probes and/or multiple templates for detection of displaced fragments, many amplification products can be tested for the presence of mismatches. Using selected probes and/or selected templates, it is possible to locate the mismatches. Using an array of probes, this can be applied to a genome wide search or to a search of multiple amplified nucleic acids simultaneously.

The use of a degenerate IP in a GMA reaction provides a means for priming the GMA at multiple sites on the template. Degeneracy of

the IP may be very high and consequently random priming of a template may be achieved. In such a case, an IP such as a random hexamer, or a longer primer with a random hexamer as its 3' sequence, is used typically with a template such as genomic DNA. More specific multiple priming may be achieve with an IP with a lower level of degeneracy.

The method according to the invention can be used as a novel signal amplification method provided by the GMA. The extended IP and the displaced fragments created in a GMA reaction have a free 3'OH terminus and can function as primers that can be extended. Using a strand displacement DNA polymerase, the displaced fragments produced during the GMA on a template are free to function as IPs in a secondary GMA reaction, if a suitable template is available. A template may be provided in the same reaction or in an uncoupled reaction as a means for signal amplification. The template provided for use in signal amplification is referred to as the signal amplification template or booster template herein. This is especially important when the amount of the original template is low, which is often the case when genomic DNA is used as the template or target nucleic acid in the initial GMA. The booster template is synthesised so that it has an intrinsic sequence complementary to any initiating primer of interest, termed 'IP binding site' herein. Typically, this sequence is complementary to the extended initiating primer or a displaced fragment produced during a GMA reaction. Typically the IP binding site is at the 3' end of the booster template (the booster template is read in a 3' to 5' direction by the polymerase that polymerises and extends the IP in a 5' to 3' direction). One or more bases are present upstream (5') of the IP binding site on the

booster template that are complementary to the modified base used in the GMA reaction. Typically, this base is an adenine residue. This results in the incorporation of a modified base, typically a U, at this position into the extending IP during the GMA reaction. The sequence following this position (upstream), referred to herein as the booster sequence is devoid of bases that are complementary to the modified base used in the initial GMA. The booster sequence can vary in size and is typically longer than 18 nucleotides. Typically, the booster sequence has a 3' modification such as an inverted nucleotide to prevent spurious self-priming. A DNA synthesis block may also be included in the booster template in the region complementary to the IP to prevent spurious self-priming. When an initiating primer primes a GMA on the booster template, it generates the complement of the booster sequence referred to as complementary booster sequence herein. The net effect of this procedure is that the complementary booster sequence is copied/amplified to a high level, as the booster template is typically not limiting, and every IP generated can potentially serve to prime any non-primed booster template in the GMA reaction. The complementary booster sequence can also serve as a universal reporter for detection purposes.

To achieve a very high level of signal amplification, the booster sequence (i.e. BS#1) can be followed by one or more bases that are complementary to the modified base in the GMA reaction. This booster sequence is followed in turn by a second booster sequence (BS#2). BS#2 is typically identical to BS#1. When an IP primes a GMA reaction on the booster template, it generates the complement of BS#1 and BS#2 referred to as complementary booster sequence #1(cBS#1) and

complementary booster sequence #2 (cBS#2) herein. cBS#1 and cBS#2 are identical and function as IPs priming the booster template from BS#1. They also bind to BS#2 but are displaced in each cycle of the GMA reaction. The net effect of this is that cBS#2 can be amplified to a high level as the booster template is typically not limiting and every cBS#1 and cBS#2 can potentially serve as initiating primers for any non-primed booster template in the GMA reaction.

There are many possibilities for booster template design. If cBS#1 is generated in the primary GMA reaction, then a booster template with only the BS#1 and BS#2 sequences, separated by a base complementary to the modified base, is necessary since the cBS#1 serves as the initiating primer. A booster template can be designed with several different IP binding sites, i.e. allowing many differently sequenced IPs to bind and prime, followed by identical or distinct booster sequence units.

A further possibility for detecting or monitoring the progression of the initiated GMA reaction is by monitoring DNA polymerase activity. Since GMA results in the continuous re-extension (i.e. polymerisation) of DNA fragments on a template, there is continuous incorporation of dNMP into the newly synthesised DNA and release of a pyrophosphate moiety (PPi) *per* each incorporated nucleotide monophosphate. Therefore, a PPi detection assay (Nyren, P. (1987) Analytical Biochemistry, 167, 235-238) can be used to indirectly detect or monitor GMA activity.

In a GMA reaction using the precursor nucleotides dATP, dCTP, dGTP, dTTP in addition to a modified precursor nucleotide such as

dUTP, the displaced fragments which are generated are of multiple sizes according to the permutations of dUTP *versus* dTTP incorporation into the newly synthesised complementary strand opposite the A residues in the template strand. A suitable IP binding site on a booster template in such a case is a sequence that is identical to a section of the initial template which is 5' (with respect to the template) of where the IP initially primed. In a sequence of DNA, a dUTP or a dTTP would be expected to be incorporated on average every fourth base since an A residue would be expected to occur at any given position in the template with a frequency of 0.25. Thus the average expected size of a displaced fragment in a GMA reaction, using dUTP in place of dTTP, would be three nucleotides. Whereas, using a ratio of dUTP to dTTP where the DNA polymerase inserts either of the two dNTPs with a 0.5 probability, the size of generated displaced fragments will range from a minimal size of three nucleotides to larger sizes where the frequency of generation of any displaced fragment decreases as the size of the displaced fragment gets larger. However, the larger the displaced fragment, the more stringent can be the hybridisation between the displaced fragment acting as an IP and the IP binding site. Thus an IP binding site on the booster template that is identical to a sequence 5' of the IP binding site on the original template nucleic acid is desirable. For example, if a displaced fragment is selected so that it has 10 positions within a 40 nucleotide segment where a dUTP or dTTP could be incorporated, an IP binding site on the booster template, complementary to the 40 nucleotides of the displaced DNA is suitable.

For detection of SNPs or mutations, the IP that primes the template nucleic acid can be positioned 3' from the chosen SNPs location with respect to the template strand, so that one permutation of the displaced fragment generated has its 3' terminus defined by the SNP site. In such a case, the presence of the SNP on the template nucleic acid leads to the generation of a displaced fragment with a unique 3'OH terminus that is not created if the SNP site is absent. The IP can be chosen so that in a GMA reaction, using a mixture of a normal and modified precursor such as dTTP and dUTP, a displaced fragment is generated of a suitable size so as to permit its hybridisation to the IP binding site on the booster template under stringent conditions. Furthermore, the IP binding site on the booster template can be designed so that it can only be primed by that displaced fragment with the unique 3'OH terminus as defined by the SNP site and the status of that site. This can be achieved by designing the booster template so that the key adenine residue supporting the GMA is sufficiently closely located 5' on the booster template so that, the 3'end of the displaced fragment generated with a 3'OH terminus defined by the next position of dUTP incorporation past the SNP site on the original template, is opposite or 5' of the key adenine residue on the booster template. Under stringent hybridisation conditions, displaced fragments where the 3'OH terminus is generated from a site upstream of the SNP site (with respect to the complement of that strand that acts as template) will not prime the booster template as they will not hybridise to the IP binding site.

According to a further embodiment of the invention an IP binding site may be degenerate so that it can serve as a binding site for many

different initiating primers. Two or more booster templates can be included in a GMA reaction so that the cBS generated from the first booster template can serve as an initiating primer for the second booster template. This has the added advantage that the second booster template can be identical for multiple different GMA reactions and thus serve as a universal booster sequence. This facilitates a single streamlined process for detection and signal amplification using a single end point booster template. Typically the end point booster template will be designed and synthesised so that it serves directly or indirectly as the reporter for the GMA.

The IP in a GMA reaction may displace a downstream extended primer that serves as an IP in a subsequent GMA reaction. This has important uses for SNP and mutation detection. A primer may be placed sufficiently close to the SNP site so that the first modified precursor incorporated is at, or distal to, the SNP site. Thus the primer is extended to a different length depending on whether or not a SNP is present at the site. It is desirable to generate multiple copies of the differentially extended primer for subsequent characterisation by any one of several means including its ability to function as an IP in a subsequent GMA on a booster template. Multiple copies of the differentially extended primer may be obtained by a thermocycling process as described for the polymerase chain reaction. Alternatively, the differentially extended primer may be repeatedly displaced by initiating a GMA reaction 5' of this primer (i.e. further 3' on the template strand). This is achieved using an IP which hybridises 5' of the primer located proximal to the SNP site and this IP initiates a GMA reaction. The downstream primer will be

extended in the same reaction in each cycle but will also be displaced in each cycle. Once displaced, fresh primer can hybridise and be extended.

Displaced fragments generated from template nucleic acids, and complementary booster sequences generated from the booster template may be detected in a 5' nuclease assay. Typically, the 5' nuclease assay is an assay that detects a specific nucleic acid by its ability to serve as an initiating primer for DNA synthesis on a template using a DNA polymerase with a 5'-3' exonuclease activity leading to degradation of a probe which is annealed downstream on the template. This degradation is based on the presence of a 5'-3' exonuclease activity in the polymerase used in the reaction. The typical probe is an oligonucleotide bearing both a reporter fluorescent dye and a quencher dye in close proximity. An increase in fluorescent intensity results when the reporter and quencher are separated / detached from each other through degradation of the probe. Thus an increase in fluorescence indicates that the probe has hybridised to the template and has been degraded by the 5' to 3' exonuclease activity of the DNA polymerase as it extends the initiating primer on the template nucleic acid.

A variation of this assay uses an approach whereby the probe is part of the template but is complementary to a section of template. This results in the probe forming a stem with part of the template by base pairing with its complement on the template nucleic acid, effectively producing a double stranded stem with one free 5' end and one looped end.

Alternatively, a self complementary probe with a reporter at one end and a quencher at the other end may be used. In this case, the probe forms a stem and loop through base pairing bringing the reporter close to the quencher so that fluorescence is quenched. Denaturation of the stem loop structure in the presence of a fully or partially complementary displaced fragment or cBS permits hybridisation between the probe and the displaced fragment or cBS. This renders the probe double stranded and increases the distance between the reporter and quencher causing an increase in fluorescent intensity.

Adaptation of the GMA for use with a linked reporter - quencher provides a unique booster template that permits simultaneous IP detection and signal amplification. Essentially, the booster template is designed with an IP binding site followed by a self complementary sequence that brings a linked reporter and quencher into close proximity through formation of a double stranded stem loop structure. The IP binding site and the sequence forming the stem - loop structure are devoid of the base complementary to the modified base in the GMA reaction. The IP binding site is 3' of the stem - loop sequence and may comprise a sequence which is complementary to an initiating primer and / or a sequence which is complementary to a cBS. One or more bases complementary to the modified base in the GMA reaction are present 5' of the stem - loop and are followed by another complementary sequence to a cBS. When an initiating primer is extended on the reporter-quencher booster template, the net effect is that the IP binding site and stem - loop section of the booster template is linearised and becomes double stranded during GMA through strand displacement and remains double

stranded. The signal is amplified through the synthesis of further cBSs at the 5' end of the booster template which in turn serve as initiating primers for any non-primed booster template in the GMA reaction. When the stem-loop is linearised and becomes double stranded, the fluorescence intensity increases and measurement of the fluorescences functions as a measure of the level on IP in the reaction.

An alternative method of signal detection in accordance with the invention involves designing the booster template so that part or all of the displaced fragment is self complementary either within itself or between other copies of itself and forms double stranded DNA. The double stranded DNA can then be detected readily by binding of double strand specific probes such as SYBR green.

The booster template may be circular. Such a circular booster template provides a means for the extending DNA polymerase to proceed continuously in a rolling circle replication format. There are multiple variations that can be used with a circular booster template. In its simplest form, the circular booster template may serve as a template for rolling circle amplification where an IP may serve as the initiator of DNA replication on the circle. In such a case, the DNA is continuously synthesised until the polymerase or the DNA precursors become limiting. Inclusion of such a booster template in a GMA reaction requires that the booster template is designed so that it does not contain any residues complementary to the modified precursor nucleotide in the GMA reaction.

Alternatively, the booster template may be designed with one or more IP binding sites followed by a residue supporting incorporation of a modified precursor on to the extending IP during GMA. This may be further followed by one or more booster sequences. The booster sequences may be preceded and followed by one or more bases that are complementary to the modified base in the GMA reaction. The complement of the booster sequence, when generated, serves as an IP for any non-primed booster templates. Priming of subsequent booster templates provides additional IP for additional non-primed booster templates and the reaction continues until all of the booster templates are primed and replication continues until one of the reagents becomes limiting. The net effect of this method is that a linear copy of all or part of the booster template is generated in each cycle. Inclusion of a second complementary booster template with a self-priming booster sequence provides a means of generating multiple linear copies of the booster template. The linear copies of the two booster templates will then hybridise to each other generating double stranded DNA which can be detected by several means, including double strand specific DNA binding agents such as SYBR green.

A single booster template may also be designed so that it produces cBSs that base pair with each other thus generating double stranded DNA. The booster template is particularly suitable for this application since regions of self complementarity within a DNA circle can be primed without denaturation by IPs hybridising to IP binding sites which are not in the self complementary region. In contrast, denaturation of fully

double stranded linear complementary DNA is necessary to allow an IP access an IP binding site.

In another embodiment of the present invention the booster or secondary template can be immobilised on a solid support, for example, on a micro- or macro-array or DNA 'chip'. Multiple different booster or secondary templates can be immobilised on a DNA chip which can then be used to characterise multiple different nucleic acids and multiple different GMA reactions in a high throughput manner. In a preferred embodiment one attaches the secondary templates *via* their 3' termini. This makes the otherwise reactive 3'OH group inaccessible, therefore it cannot be extended by a polymerase and acts only as a template, thereby reducing any background non-specific extension.

Another embodiment of the present invention provides for the use of GMA in DNA computing. It is now increasingly appreciated that DNA, by virtue of its intrinsic physical and chemical properties, may provide an avenue for the computation of solutions to difficult mathematical tasks. It will be appreciated that by designing initiating primers, templates and/or booster templates to act as individual or combined pieces of information, be it problems and/or solutions, the solutions to mathematical problems can be computed using the GMA reaction. Preferably, the IPs and templates are artificially synthesised. It will also be appreciated that the use of GMA in DNA computing permits simultaneous operations and parallel searches and can give rise to a complete set of potential solutions.

Accordingly, the invention provides use of a method as hereinbefore described in DNA computing and/or as a "tool" in DNA computing, referred to herein collectively as DNA computing.

The invention will be further illustrated by the following Examples.

Modes for Carrying Out the Invention

Various enzymes were used in the following Examples. Some of these enzymes were available commercially while others were purified from over-expression in *E. coli* strains as described below.

Thermotoga maritima UDG (TmaUDG)

The open reading frame for the TmaUDG protein was amplified from *T. maritima* genomic DNA by PCR. The PCR product was inserted into the pBAD-TOPO over-expression vector according to the manufacturer's instructions (InVitrogen). Competent TOP-10 *E. coli* (InVitrogen) were then transformed with the construct by heat shock, according to the manufacturer's instructions. Cells containing the pBAD-TOPO/TmaUDG construct were grown to an OD_{600} of 0.6 (1000mL) and over-expression was induced by arabinose added to a final concentration of 0.2%. After incubation at 37°C for 4hours the cells were lysed and the fusion protein was purified by immobilised metal affinity chromatography using ProBond resin (Invitrogen) according to the manufacturer's instructions. A 15mL fraction containing the eluted protein as judged by SDS-polyacrylamide gel electrophoresis was

collected. The fraction was then further purified by ion-exchange chromatography using Mono-S resin and 0.5mL of the most concentrated eluted protein fraction was collected and stored after addition of glycerol to 50% at –20°C until required.

Thermotoga maritima Endonuclease IV (TmaEndoIV)

The open reading frame for the TmaEndoIV protein was amplified from *T. maritima* genomic DNA by PCR. The PCR product was inserted into the pBAD-TOPO over-expression vector according to the manufacturer's instructions (InVitrogen). Competent TOP-10 *E. coli* (InVitrogen) were then transformed with the construct by heat shock, according to the manufacturer's instructions. Cells containing the pBAD-TOPO/TmaEndoIV construct were grown to an OD_{600} of 0.6 (1000mL) and over-expression was induced by arabinose added to a final concentration of 0.2%. After incubation at 37°C for 4hours the cells were lysed and the fusion protein was purified by immobilised metal affinity chromatography using ProBond resin (Invitrogen) according to the manufacturer's instructions. A 15mL fraction containing the eluted protein as judged by SDS-polyacrylamide gel electrophoresis was collected. The fraction was then further purified by ion-exchange chromatography using Mono-S resin and 0.5mL of the most concentrated eluted protein fraction was collected and stored after addition of glycerol to 50% at –20°C until required.

Thermotoga maritima Endonuclease V (TmaEndoV)

The open reading frame for the TmaEndoV protein was amplified from *T. maritima* genomic DNA by PCR. The PCR product was inserted into the pBAD-TOPO over-expression vector according to the manufacturer's instructions (InVitrogen). Competent TOP-10 *E. coli* (InVitrogen) were then transformed with the construct by heat shock, according to the manufacturer's instructions. Cells containing the pBAD-TOPO/TmaEndoV construct were grown to an OD_{600} of 0.5 (1000mL) and over-expression was induced by arabinose added to a final concentration of 0.2%. After incubation at 37°C for 8hours the cells were lysed and the fusion protein was purified by immobilised metal affinity chromatography using ProBond resin (Invitrogen) according to the manufacturer's instructions. 15 x 1mL fractions containing the eluted protein as judged by SDS-polyacrylamide gel electrophoresis were collected and pooled. The pooled fractions were then further purified by ion-exchange chromatography using Mono-S resin and 0.5mL of the most concentrated eluted protein fraction was collected and stored after addition of glycerol to 50% at –20°C until required.

E. coli Endonuclease IV (EcoEndoIV)

The open reading frame for the EcoEndoIV protein was amplified from *E. coli* genomic DNA by PCR. The PCR product was inserted into the pBAD-TOPO over-expression vector according to the manufacturer's instructions (InVitrogen). Competent TOP-10 *E. coli* (InVitrogen) were

then transformed with the construct by heat shock, according to the manufacturer's instructions. Cells containing the pBAD-TOPO/ EcoEndoIV construct were grown to an OD_{600} of 0.6 (1000mL) and over-expression was induced by arabinose added to a final concentration of 0.2%.

After incubation at 37°C for 4hours the cells were lysed and the fusion protein was purified by immobilised metal affinity chromatography using ProBond resin (Invitrogen) according to the manufacturer's instructions. A 15mL fraction containing the eluted protein as judged by SDS-polyacrylamide gel electrophoresis was collected. The pooled fractions were then further purified by ion-exchange chromatography using Mono-S resin and 0.5mL of the most concentrated eluted protein fraction was collected and stored after addition of glycerol to 50% at –20°C until required.

Archaeoglobus fulgidus UDG-Thioredoxin (AfUDG-Thio)

The open reading frame for the AfUDG protein was amplified from *A. fulgidus* genomic DNA by PCR. The PCR product was inserted into the pBAD-TOPO ThioFusion over-expression vector according to the manufacturer's instructions (InVitrogen). Competent TOP-10 *E. coli* (InVitrogen) were then transformed with the construct by heat shock, according to the manufacturer's instructions. Cells containing the pBAD-TOPO-ThioFusion/AfUDG construct were grown to an OD_{600} of 0.6 (2000mL) and over-expression was induced by arabinose added to a

final concentration of 0.2%. After incubation at 37°C for 4hours the cells were lysed and the fusion protein was purified by immobilised metal affinity chromatography using ProBond resin (Invitrogen) according to the manufacturer's instructions. A 15mL fraction containing the eluted protein as judged by SDS-polyacrylamide gel electrophoresis was collected and stored at 4°C until required.

Example 1

The method according to the invention was used to cyclically extend a 25-mer oligonucleotide (Initiating Primer-IP), which was complementary to a region of an 80-mer oligonucleotide (Template nucleic acid) which served as the template for the polymerisation reaction. The complementary region extended from 10 bases from the 3' end of the 80-mer to 35 bases from the 3' end of the 80-mer. The region of the 80-mer 5' to this IP complementary region (IP binding site) was designed to contain a number of A residues which upon cyclical extension of the 25-mer would lead to the production of DNA fragments of discrete sizes following the GMA reaction. The 80-mer was also designed so that a specific number of G residues lay between each A residue. This was to allow labelling, by incorporation of α^{32}P-dCTP, of the displaced downstream DNA fragments produced during the reaction. A flow diagram of the method is shown in Fig. 1. Both oligonucleotides were synthesised artificially and purified by excision from a polyacrylamide gel after electrophoresis. The objective was to determine if the 25-mer annealed to the 80-mer and could be cyclically/repeatedly

extended by the method according to the invention, producing multiple copies of labelled single stranded DNA fragments (displaced fragments).

Before addition of the enzymes, the reactions contained 100fmole of each of the oligonucleotides, 10mM Tris-HCl (pH7.5), 5mM $MgCl_2$, 7mM dithiothreitol, 0.2mM each of dATP, dGTP and dUTP, 0.02mM dCTP and 0.2μL α^{32}PdCTP (10mCi/ml, ~800Ci/mmol) in a total of 17μL. This mixture was overlaid with mineral oil and heated to 95°C for 2min. The reaction temperature was then dropped to 37°C and held at this temperature. Five units of Klenow Fragment (3' → 5' exo$^-$), 1 unit of *E. coli* Uracil DNA Glycosylase and 2 units of either *E. coli* Endonuclease IV or 2μL *Thermotoga maritima* Endonuclease IV were then added, in that order, bringing the final reaction volume to 20μL. In the case of the control reactions, which contained no Endonuclease IV and the control reactions that contained neither Endonuclease IV nor Uracil DNA Glycosylase, water was added to bring the final volume to 20μL. 5μL samples were taken from the reactions at 30min intervals after the addition of the Endonuclease IV. NaOH was added to the samples to a final concentration of 50mM and then heated to 95°C for 15min. An equal volume of formamide loading dye (98% formamide, 0.025% Bromophenol blue, 0.025% Xylene cyanol) was then added. The samples were then loaded onto a 20% denaturing (7M urea) polyacrylamide gel and electrophoresis was carried out for size analysis of the DNA fragments. Following electrophoresis, the gel was exposed to a phosphor screen and the image was subsequently scanned by a Storm (Trade Mark) 860.

Analysis of the image showed that the reactions which contained Endonuclease IV contained a number of labelled DNA fragments of different sizes shorter than 70 bases, which increased in number with time, and which did not appear in the control reactions which lacked the Endonuclease IV, or in the control reaction which lacked both Endonuclease IV and Uracil DNA Glycosylase.

It was noted that the *Thermatoga maritima* Endonuclease IV preparations contained an intrinsic and/or contaminating 3' to 5' exonuclease activity. This lead to non-specific background amplification during a GMA reaction. To eliminate this activity and non-specific amplification, the Endonuclease IV enzyme was heat treated prior to its use in the GMA reaction. The stock of Endonuclease IV enzyme was heated to 90°C for 10 min, then put on ice for ≥ 5 min and subsequently centrifuged for ≥ 5 min. The *E. coli* Endonuclease IV preparations were also observed to contain such an activity and the preparations also required inactivation of the exonuclease activity prior to use.

Example 2

The method according to the invention was used to cyclically extend a 41-mer oligonucleotide (Initiating Primer-IP) which was self-complementary at its 3' end, i.e. palindromic, and therefore served as the template for itself for the GMA reaction. The complementary region extended for 20 bases from the 3' end of the 41-mer and this section of the oligonucleotide esssentially acted as the IP. The region of the 41-mer 5' to this IP complementary region (IP binding site) was designed to contain one A residue following the IP binding site, which upon cyclical

extension of the 41-mer would lead to the production of a discrete DNA fragment of 20 nucleotides following the GMA reaction. The 41-mer was also designed so that it contained a number of G residues. This was to allow labelling, by incorporation of α^{32}P-dCTP, of the displaced downstream DNA fragments produced during the reaction (20-mer). The oligonucleotide was synthesised artificially and purified by excision from a polyacrylamide gel after electrophoresis. The objective was to determine if the 41-mer annealed to itself and could be cyclically/repeatedly extended by the method according to the invention, producing multiple copies of the labelled single stranded 20-mer DNA fragment (displaced fragment).

Before addition of the enzymes, the reactions contained 100fmole of the 41-mer oligonucleotide, 10mM Tris-HCl (pH7.5), 5mM $MgCl_2$, 7mM dithiothreitol, 0.2mM each of dATP, dGTP and dUTP, 0.02mM dCTP and 0.2μL α^{32}PdCTP (10mCi/ml, ~800Ci/mmol) in a total of 17μL. This mixture was overlaid with mineral oil and heated to 95°C for 2min. The reaction temperature was then dropped to 37°C and held at this temperature. Five units of Klenow Fragment (3' → 5' exo$^-$), 1 unit of *E. coli* Uracil DNA Glycosylase and 2 units of either *E. coli* Endonuclease IV or 2μL *Thermotoga maritima* Endonuclease IV were then added, bringing the final reaction volume to 20μL. In the case of the control reactions which contained no Endonuclease IV and the control reactions which contained neither Endonuclease IV nor Uracil DNA Glycosylase, water was added to bring the final volume to 20μL. The reaction was allowed to proceed for greater than or equal to 30 min. EDTA was added to a final concentration of 10mM to stop the reaction.

An equal volume of formamide loading dye (98% formamide, 0.025% Bromophenol blue, 0.025% Xylene cyanol) was then added. The samples were then loaded onto a 20% denaturing (7M urea) polyacrylamide gel and electrophoresis was carried out for size analysis of the DNA fragments. Following electrophoresis, the gel was exposed to a phosphor screen and the image was subsequently scanned by a Storm 860 (Storm is a Trade Mark).

Analysis of the image showed that a detectable quantity of the labelled and amplified 20-mer oligonucleotide was produced in each test reaction, but not in the control reactions.

Example 3

The method according to the invention was carried out as described in Example 2 with some modifications, as follows:

Before addition of the enzymes the reaction contained 100fmole 41-mer palindromic oligonucleotide, 10mM Tris-HCl (pH 8.3) 10mM KCl, 3mM $MgCl_2$, 0.2mM each of dATP, dUTP and dGTP, 0.02mM dCTP and α-^{32}P-dCTP in a volume of 20µL. This mixture was overlaid with mineral oil and heated to 95°C for 2min. The temperature was then dropped to 60°C and held there. 10 units of AmpliTaq (Trade Mark) DNA Polymerase Stoffel Fragment, 2µL units *Thermotoga maritima* UDG and 4 units *E. coli* Endonuclease IV (or water in the case of the control reaction containing no Endonuclease IV) were added to the reaction in that order, to bring the final reaction volume to 25µL. 5µL samples of the reactions were taken at 30min intervals after the addition

of the Endonuclease IV. These samples were brought to a final concentration of 10mM EDTA. An equal volume of formamide loading dye (98% formamide, 0.025% Bromophenol blue and 0.025% Xylene cyanol) was then added. Size analysis of the DNA samples was carried out as in Example 1.

Analysis of the image showed that the reactions containing Exonuclease IV contained large amounts of labelled DNA, which was absent from the control reactions which lacked the Endonuclease IV. The amount of DNA in the test reactions increased with time reaching a peak at 90min from the addition of the Endonuclease IV.

Example 4

The method according to the invention was carried out as described in Example 2 with some modifications, as follows

Before addition of the enzymes the reaction contained 100fmole of the 41mer oligonucleotide, 10mM Tris-HCl (pH 8.3) 10mM KCl, 3mM $MgCl_2$, 0.2mM each of dATP, dUTP and dGTP, 0.02mM dCTP and 0.2μL α-^{32}P-dCTP (10mCi/ml, ~800Ci/mmol) in a volume of 20μL. This mixture was overlaid with mineral oil and heated to 95°C for 2min. The temperature was then dropped to 70°C and held there. 10 units of AmpliTaq DNA Polymerase Stoffel Fragment, 2μL AfUDG-Thio and 2μL EcoEndoIV (or water in the case of the control reaction containing no Endonuclease IV) were added to the reaction in that order, to bring the final reaction volume to 25μL. The reaction was terminated after 60min by the addition of EDTA to a final concentration of 10mM. An

equal volume of formamide loading dye (98% formamide, 0.025% Bromophenol blue and 0.025% Xylene cyanol) was then added. Size analysis of the DNA samples was carried out as in Example 1. Following electrophoresis, the gel was exposed to a phosphor screen and the image was detected using a Storm (Trade Mark) 860 phosphoimager.

Analysis of the image showed that the reactions containing Exonuclease IV contained large amounts of the expected labelled 20mer and to a lesser extent, some smaller fragments, which were absent from the control reactions in the absence of added Endonuclease IV.

Example 5

The method according to the invention was used to detect primer extension from attomole quantities of the 41-mer palindromic oligonucleotide. Uracil DNA Glycosylase digestion followed by Endonuclease IV digestion of the product formed by the polymerase catalysed extension of the 41-mer oligonucleotide using itself as template yields as one of its products a 20-mer single stranded oligonucleotide (i.e. displaced fragment which can subsequently act as an IP). This oligonucleotide acts as a secondary primer. In this example the method according to the invention allows the detection of the cyclical extension of the 20-mer oligonucleotide through a feedback loop reaction. A 42-mer oligonucleotide (booster template) was designed as schematically represented in Fig. 2. The complementary sequence of the 20-mer secondary primer/displaced fragment is repeated twice in this 42-mer. The repeated sequences are separated (reading 5' to 3') by an AT

sequence. This oligonucleotide was synthesised and purified in the same way as the 41-mer palindromic oligonucleotide. This 42-mer was included in large excess in the reaction. Once the 20-mer secondary primer has been produced as described above, it can then anneal to the 42-mer oligonucleotide and, by the method according to the invention, be itself cyclically extended, producing as a result numerous copies of itself. These newly produced 20-mer secondary primers can then themselves anneal to other copies of the 42-mer oligonucleotide initiating new rounds of cyclical extension and so on. The objective was to detect quantities of the 41-mer palindromic oligonucleotide, normally too small to detect as described in Example 2, by the production of detectable quantities of the 20-mer secondary primer through the feedback loop application of the method according to the invention described above.

The reactions described in Example 2 were repeated in triplicate except for the following modifications. The 42-mer described above was included in each reaction to a final concentrations of 40ng/reaction. The concentration of the 41-mer palindromic oligonucleotide was 1.0 fmole, 10^{-2} fmole or 10^{-4} fmole *per* reaction in the different sets of control (no Endonuclease IV included in the reaction) and test reactions (Endonuclease IV included in the reaction). The reactions were allowed to run for 2 hours before the addition of the NaOH to a final concentration of 50mM. The labelled DNA fragment content of the reactions was analysed by analysis of the equivalent of 5μL of the original reactions (before addition of the NaOH and formamide loading dye) as described in Example 1.

Analysis of the image showed that a detectable quantity of the labelled 20-mer oligonucleotide was produced in each test reaction, but not in the control reactions.

A similar result was observed when the above experiment was repeated using the thermostable enzymes, DNA Stoffel Fragment, *Thermatoga maritima* UDG and *Thermatoga maritima* Endonclease IV. To avoid any non-specific amplification arising from the self- priming of the booster template, the 3' end of the booster usually contains a blocking group which prevents extension by DNA polymerase. The blocking group used on the 42-mer booster described above was a dideoxycytosine nucleotide.

Example 6

The method according to the invention was used to cyclically extend a self-annealing 41mer. This reaction produced a 20mer that could then act as an initiation primer on a 42mer (booster) with a blocked 3' terminus (the block used was an inverted cytidine nucleotide). The booster was designed such that this secondary reaction would also produce the same 20mer by cyclical extension (see Fig. 2). These 20mers could then prime further extension on other booster molecules, amplifying the signal from the original reaction on the 41mer. The 41mer and 42mer booster were designed to allow the incorporation of radio-labelled α^{32}PdCTP. Reactions contained either (1) 100fmole of the 41mer, (2) 1fmole of the 41mer, (3) 100fmole of booster or (4) 1fmole of the 41mer and 100fmole of a booster in Taq Stoffel Fragment buffer (10mM Tris-HCl, 10mM KCl, pH8.3) supplemented with 3mM $MgCl_2$,

0.2mM dATP, 0.2mMdGTP, 0.2mM dUTP and 0.02mM and 0.2uL α^{32}PdCTP (10mCi/ml, ~800Ci/mM). This reaction mix was overlaid with oil and incubated at 95°C for 2min before being brought to and maintained at 70°C. 10U Taq Stoffel Fragment, 2μL AfUDG-Thio and 2μL EcoEndoIV were then added in that order, bringing the final reaction volume to 25μL. The reaction was incubated at 70°C for 60min and then terminated by the addition of EDTA to a final concentration of 10mM. An equal volume of 98% formamide loading buffer (containing 0.025% Bromophenol blue, 0.025% Xylene cyanol) was added and samples were analysed by denaturing 20% polyacrylamide gel electrophoresis. Following electrophoresis, the gel was exposed to a phosphor screen and the image was detected using a Storm (Trade Mark) 860 phosphoimager.

Analysis of the image showed that in reaction (1) there was production of a 20mer labelled DNA product, and to a lesser extent a ~60mer and a number of products smaller than the 20mer. The same product was produced in reaction (2) but to a far lesser extent. There was no labelled reaction product in reaction (3) however in reaction (4) a labelled 20mer was produced which was more intense than that produced in reaction (2) as well as to a lesser extent a number of labelled products smaller than the 20mer. It was observed that the use of inverted nucleotides as 3' terminal blocks was more effective than use of dideoxynucleotides as terminal blocks.

Example 7

The method according to the invention was used to cyclically extend a 41-mer oligonucleotide (Initiating Primer-IP), which was self-complementary at its 3' end, i.e. palindromic, and therefore served as the template for itself for the GMA reaction. The complementary region extended for 20 bases from the 3' end of the 41-mer and this section of the oligonucleotide essentially acted as the IP. The region of the 41-mer 5' to this IP complementary region (IP binding site) was designed to contain one A residue following the IP binding site, which upon cyclical extension of the 41-mer would lead to the production of a discrete DNA fragment of 20 nucleotides following the GMA reaction. The 41-mer was also designed so that it contained a number of G residues. This was to allow labelling, by incorporation of α^{32}P-dCTP, of the displaced downstream DNA fragments produced during the reaction (20-mer). The oligonucleotide was synthesised artificially and purified by excision from a polyacrylamide gel after electrophoresis. The objective was to determine if the 41-mer annealed to itself and could be cyclically/repeatedly extended by the method according to the invention with *E. coli* Exonuclease III in place of *E. coli* endonuclease IV, producing multiple copies of the labelled single stranded 20-mer DNA fragment (displaced fragment). Before addition of the enzymes, the reactions contained 100fmole of the 41-mer oligonucleotide, 10mM Tris-HCl (pH7.5), 5mM $MgCl_2$, 7mM dithiothreitol, 0.2mM each of dATP, dGTP and dUTP, 0.02mM dCTP and 0.2μL α^{32}PdCTP (10mCi/ml, ~800Ci/mmol) in a total of 22μL. This mixture was overlaid with mineral oil and heated to 95°C for 2min. The reaction temperature was

then dropped to 37°C and held at this temperature. Five units of Klenow Fragment (3' → 5' exo$^-$), 10 units of *E. coli* Uracil DNA Glycosylase and 0.1 units of *E. coli* exonuclease III were then added, bringing the final reaction volume to 25μL. In the case of the control reactions which contained no Exonuclease III, water was added to bring the final volume to 25μL. The reaction was allowed to proceed for greater than or equal to 30 min. EDTA was added to a final concentration of 10mM to stop the reaction. An equal volume of formamide loading dye (98% formamide, 0.025% Bromophenol blue, 0.025% Xylene cyanol) was then added. The samples were then loaded onto a 20% denaturing (7M urea) polyacrylamide gel and electrophoresis was carried out for size analysis of the DNA fragments. Following electrophoresis, the gel was exposed to a phosphor screen and the image was detected using a Storm (Trade Mark) 860 phosphoimager.

Analysis of the image showed that a detectable quantity of the labelled 20-mer oligonucleotide was produced in each test reaction, but not in the control reactions.

Example 8

The method according to the invention was used to cyclically extend a 21-mer oligonucleotide (Initiating Primer-IP), which was complementary to a region of a 40-mer oligonucleotide (template nucleic acid) which served as the template for the polymerisation reaction. The complementary region extended from the 3' end of the 40-mer. The region of the 40-mer 5' to this IP complementary region (IP binding site)

was designed to contain a number of C residues which upon cyclical extension of the extended 21-mer would lead to the production of DNA fragments of discrete sizes following the GMA reaction. The 40-mer was also designed so that a number of G residues lay between each A residue. This was to allow labelling, by incorporation of α^{32}P-dCTP, of the displaced downstream DNA fragments produced during the reaction. Both oligonucleotides were synthesised artificially and purified by excision from a polyacrylamide gel after electrophoresis. The objective was to determine if the 21-mer annealed to the 40-mer and could be cyclically/repeatedly extended by the method according to the invention, producing multiple copies of labelled single stranded DNA fragments (displaced fragments). Before addition of the enzymes, the reactions contained 100fmole of each of the oligonucleotides, 10mM Tris-HCl (pH7.5), 6mM $MgCl_2$, 10mM KCl, 0.2mM each of dATP, dITP and dTTP, 0.02mM dCTP and 0.2μL α^{32}PdCTP (10mCi/ml, ~800Ci/mmol) in a total of 22μL. This mixture was overlaid with mineral oil and heated to 95°C for 2min. The reaction temperature was then dropped to 70°C and held at this temperature. Ten units of Taq polymerase Stoffel Fragement, and 800pg of TmaEndoV was then added, in that order, bringing the final reaction volume to 25μL. In the case of the control reactions, which contained no Endonuclease V, water was added to bring the final volume to 25μL. Reactions were terminated by the addition of EDTA to a final concentration of 10mM. One half volume of formamide loading dye (98% formamide, 0.025% Bromophenol blue, 0.025% Xylene cyanol) was then added and the reaction products denatured by heating to 95°C for 5min. The samples were then loaded onto a 20% denaturing (7M urea) polyacrylamide gel and electrophoresis was carried

out for size analysis of the DNA fragments. Following electrophoresis, the gel was exposed to a phosphor screen and the image was detected using a Storm (Trade Mark) 860 phosphoimager.

Analysis of the image showed that the reactions, which contained Endonuclease V, contained a number of labelled DNA fragments of different sizes which were delineated by the relevant position of dITP incorporation in the extension, and which increased in number with time, and which did not appear in the control reactions that lacked the Endonuclease V.

Claims: -

1. A method for the amplification of a template nucleic acid, which comprises simultaneously carrying out the steps of:

i) reacting a nucleic acid primer with said template nucleic acid, normal DNA precursor nucleotides, at least one modified DNA precursor nucleotide and a DNA polymerase so as to obtain an extended nucleic acid primer, said nucleic acid primer remaining bound to said template;

ii) cleaving the modified base-containing extended nucleic acid primer so as to generate a free 3'-OH terminus that is extendible by said DNA polymerase; and

iii) repeating steps i) and ii) on DNA fragments thereby generated.

2. A method according to Claim 1, wherein the modified base-containing extended nucleic acid primer is cleaved by a 3'-endonuclease.

3. A method according to Claim 2, wherein the 3'-endonuclease is Endonuclease V from *E. coli*.

4. A method according to Claim 1, which comprises the steps of :

i) reacting a nucleic acid primer with said template nucleic acid, normal DNA precursor nucleotides, at least one

modified DNA precursor nucleotide which is a substrate for a DNA glycosylase, and a DNA polymerase so as to obtain an extended nucleic acid primer, said nucleic acid primer remaining bound to said template;

ii) excising the modified base of the modified DNA precursor nucleotide from the extended nucleic acid primer by means of a DNA glycosylase so as to generate an abasic site;

iii) cleaving the extended nucleic acid primer at the abasic site so as to generate a free 3'-OH terminus that is extendible by said DNA polymerase; and

iv) repeating steps i)-iii) on DNA fragments thereby generated.

5. A method according to any preceding claim, wherein the template nucleic acid is DNA.

6. A method according to any preceding claim, wherein the nucleic acid primer is a DNA primer.

7. A method according to any one of Claims 1-6, wherein the DNA precursor nucleotides are selected from dATP, dCTP, dGTP and dTTP.

8. A method according to any preceding claim, wherein the DNA polymerase has strand displacement activity.